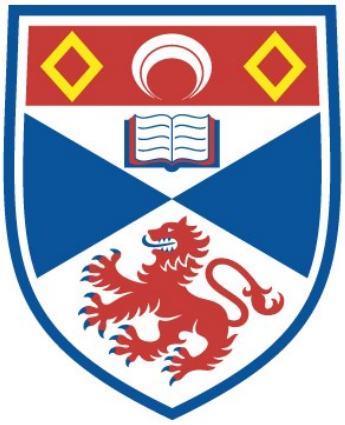
BIOCHEMICAL ABNORMALITIES IN ERYTHROCYTES FROM PATIENTS WITH DUCHENNE MUSCULAR DYSTROPHY AND MULTIPLE SCLEROSIS

Mio Sam Lao

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



1984

BIOCHEMICAL ABNORMALITIES IN ERYTHROCYTES FROM PATIENTS WITH DUCHENNE MUSCULAR DYSTROPHY AND MULTIPLE SCLEROSIS

by

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A thesis submitted to the
University of St. Andrews in
application for a degree
of Doctor of Philosophy

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August 1983

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DECLARATION

I hereby declare that the following thesis is based on work carried out by me, that the thesis is my own composition and that no part of it has been presented previously for a higher degree.

The research was carried out in the Department of Biochemistry and Microbiology of the University of St. Andrews under the direction of Dr. M.I.S. Hunter.

CERTIFICATE

I hereby declare that Mio Sam Lao has spent nine terms in research work under my supervision and that she has fulfilled the conditions of Ordinance No. 16 (St. Andrews) and that she is qualified to submit this thesis for the degree of Doctor of Philosophy.

ACADEMIC RECORD

I matriculated at Hong Kong Baptist College in September, 1974 and graduated with a Diploma (Scholastic Award) in the Department of Biology in June, 1978.

In October, 1979, I matriculated as a research student at the University of St. Andrews.

ACKNOWLEDGEMENTS

I would like to thank my Supervisor, Dr. M.I.S. Hunter, for his encouragement and advice throughout this work; the Maitland Ramsay Trust for the financial support; Mr. Bill Blyth for all the photographic work; Mr. D. Kirk for his technical assistance; friends for donating blood and Miss M. Wilson for typing this thesis.

DEDICATION

To my Parents.

The aim of science is to seek the simplest explanation of complex facts.

- Alfred North Whitehead

CONTENTS

CHAPTER I

DUCHENNE MUSCULAR DYSTROPHY

1. INTRODUCTION

1.1 Genetic Status

Duchenne Muscular Dystrophy (DMD) is an X-linked recessive lethal disease with a high incidence of 1 in 3067 male births (Gardner-Medwin, 1970).

Davie (1978) and Caskey, Nussbaum, Cohan and Pollack (1980) proposed that 1/3 of all cases of X-linked DMD are new mutants, the remainder being sons of carriers, by using the creatine phosphokinase test (cpk test) as carrier detection method. But Ionasescu and Hanson (1980) and Roses and Appel (1976) hold a different opinion. Ionasescu et al. examined ribosomal protein synthesis in muscle of DMD carriers and non carriers (mothers of isolated cases), and this study revealed that the new mutation rate was much smaller than the 1/3 value. Roses et al. (1976) found the endogenous phosphorylation of peak II (on SDS electrophorogram of erythrocyte membrane proteins) was significantly increased in definite, probable and possible carriers while some of these carriers had normal serum cpk activity. They concluded the cases previously considered to be new mutationsare much less common than estimated. However, no firm conclusions about the mutation rate of DMD can be drawn at the present time.

1.2 Symptoms and Diagnostic Signs

DMD is the most severe form of muscular dystrophies. The affected boys show clinical symptoms as early as 2-3 years of age. First they show clumsiness in walking and are unable to run properly. At four years old, they show a waddling gait, delayed psychomotor development and sometimes immaturity of speech. They also show hypotonia of shoulder muscle and pseudohypertrophy of the calf muscle. This also occurs in about 8% of carriers. Weakness of individual muscles progresses to shoulder, arm and pelvic girdle. The muscle becomes pseudohypertrophic as the muscle fibres are replaced by fat and fibrous tissue. The mean age at which there is inability to walk is about 9.5 years but this may vary from about seven to fourteen years. Spinal deformity may develop in most cases within a few years after they become unable to walk.

The electrocardiogram is abnormal in almost all patients. Some patients may have mental retardation, the features of which can not be attributed to effects of primary muscle pathology. Weakness of the paraspinal muscles contributes to the eventual thoracic distortion and this in turn is a major factor in the chronic respiratory insufficiency from which most patients die, usually in their early twenties (Gardner-Medwin, 1980).

The microscopic morphology of muscle degeneration and serum changes in the disease are discussed in the following sections. Since carriers may exhibit similar

symptoms, although to a lesser extent, both patients and carriers will be discussed together.

1.2.1 Muscle Necrosis

According to Cullen and Fulthorpe (1975) fibre breakdown in DMD can be divided into five stages by electron microscopical examination.

Stage 1 The fibres appear normal, but the volume fraction of the mitochondria was less than normal. The amount of sarcoplasm is clearly higher than in healthy fibres.

Stage 2 Fibres were overstretched, caused by strong overcontraction of the sarcomeres in other areas of the same fibres. Sarcoplasmic reticulum, parts of which are dilated, loses its regular distribution. Mitochondria are similarly displaced.

Stage 3 Localized contraction has continued to the extent that the stretched areas between the clumped myofibrils are partly empty of contractile material. Isolated segments of myofibril are scattered between the contracted areas. The greater part of the interstitial areas is occupied by sarcoplasm containing empty vesicles, degenerating mitochondria, glycogen particles and occasional lipid droplets.

Stage 4 The clumps of contractile material are further condensed and their outlines are more rounded. Around the contraction clumps are large areas of granular sarcoplasm which contain little apart from occasional vesicles or mitochondria.

Stage 5 The fibre, which is progressively invaded by macrophages, now consists only of structurless cytoplasm containing no contractile material. Occasionally regenerating fibres are found in the vicinity of these final remnants.

Regeneration of muscle fibres has been shown to compensate for degenerated fibres. This has been suggested by the increased turnover of muscle contractile proteins assessed by 3-methyhistidine and creatine excretion (Ballard, 1979). However, the regeneration does not fully compensate for necrosis. The regenerating fibres do not always attain normal size indicating an endogenous alteration in the muscle. Other fibres are thus able to hypertrophy and the number of muscle cells decreases. Collagen progressively accumulates in dense bundles lying between and parallel to the muscle cells, presumably as a sequel of necrosis when regeneration fails to take place (Carpenter and Karpati, 1979).

1.2.2 Serum Changes

Almost all of the enzyme activities that are increased in serum in DMD patients and carriers are normally cytoplasmic, or only loosely attached to organelles or myofilaments. Enzymes that are bound to membranes or other structures in muscle are not ordinarily found in the plasma of DMD patients (Rowland, 1980). Not only enzymes from skeletal muscles, but also those from heart, liver and erythrocytes were found to be increased in serum, indicating a generalised enzyme release.

Some enzymes increased in serum of DMD patients and carriers are summarized in Table 1.

The increased serum enzyme activities return toward normal values when the boys grow older and, concomitantly, muscle mass gradually diminishes (Dreyfus, Schapira and Demos, 1958).

Non-enzymic proteinswere also found to be increased in the serum of DMD patients and carriers. They are summarized in Table 2.

1.2.3 Urinary Excretion Changes

Some breakdown products of muscle are usually found in DMD urine.

1. Creatine and creatinine

Creatinine is found in most muscle disease including DMD. These changes are primarily a non-specific consequence of muscle wasting (Pennington, 1980).

TABLE 1 Soluble muscle specific enzymes found in DMD serum

Enzymes	Isoenzyme Pattern		Remarks	Refs.
	Patients	Carriers		
creatine phosphokinase (cpk)	MM (muscle) MB (cardic)	MM faint MB	resemble fetal muscle	Yasmineh, 1978; Rowland, 1980
Aldolase	muscle type	muscle type		Schapira, Dreyfus and Schapira, 1957
Lactate dehydrogenase (LDH)	LDH-5 reduced	LDH-5 increased		Somer, Donner, Murros and Konttineus, 1973
Pyruvate kinase (pk)				Zatz and Otto, 1980

TABLE 2 Non-enzymatic protein reported as being elevated in serum of DMD patients

Protein	Remarks	References
Myoglobin	not specific to muscular dystrophies	Adornato, Kagen and Engel, 1978
Hemopexin	as a carrier to fix the increased myoglobin released from muscles	Askanas, 1966; Danieli and Angelini, 1976
α_2-macroglobulin	plasma inhibitor of the muscle lyosomal protease, Cathepsin B, suggested to be a counterbalance of increased protein catabolism in DMD muscle	Jones, 1982

Creatinine excretion was also found to be decreased (Kobayashi, Shinnok and Mawatari, 1979). These changes might be due to the reduced muscle mass, much of the creatine having escaped from uptake into the muscles and conversion to creatinine (Pennington, 1980).

2. Amino acids

Taurine, proline, ornithine, 3-methyl-histidine and dimethylarginines were found to be increased in DMD urine (Bank, Rowland and Ipsen, 1979; Lou, 1979). These results indicated a relation between protein methylation processes and muscle dystrophies.

3. Polyamines

Russel and Stern (1981) found increases in polyamines, putrescine, spermidine and spermine in DMD patient urine. The marked increase of urinary polyamines in DMD may result from altered intracellular accumulation patterns of polyamines in dystrophic muscle.

4. Proteins

Two additional, unidentified proteins were found in the urine in DMD patients and 60% of carriers but in none of the controls (Fearson, Taylor and Percy, 1981). They suggested that these might be a consequence of muscle damage.

To summarize, most of the seral and urinary changes in DMD patients and carriers are secondary symptoms of muscle wasting. They can be a diagnostic tool and form the basis of carrier detection methods but probably tell us little about the primary defect of DMD.

1.3 Theories for the Pathogenesis of DMD

1.3.1 Neurogenic and Vascular Hypotheses

McComas, Sica and Upton (1974) demonstrated a progressive reduction in the number of functioning motor units. The motor neurons were referred to as "'sick', a state characterized by difficulty in maintaining satisfactory synaptic connections with muscle fibres". The theory was postulated that muscular dystrophy is a chronic dysfunction of motor neurons, eventually leading to their physiological failure. But McComas' theory could not explain the increase of cpk in DMD patients and carriers, the different serum enzyme patterns and the cardiac abnormality in DMD. The neurogenic theory seems to be fading nowadays.

The vascular hypothesis was first proposed by Kunè and Okimaka, 1930. They suggested that inadequate blood flow might account for the degeneration of dystrophic muscle. But this simple idea was abolished by the finding of Paulson, Engel and Jomez (1974). They found that blood flow at rest and during the hyperemia which follows exercise was normal and capillary diffusion capacity was unimpaired. No other evidence of a structural or functional defect in the microcirculation of DMD patients was found.

1.3.2 Membrane Theory

This is the most popular and widely accepted theory to explain the primary defect of DMD, but encompasses a large number of variants.

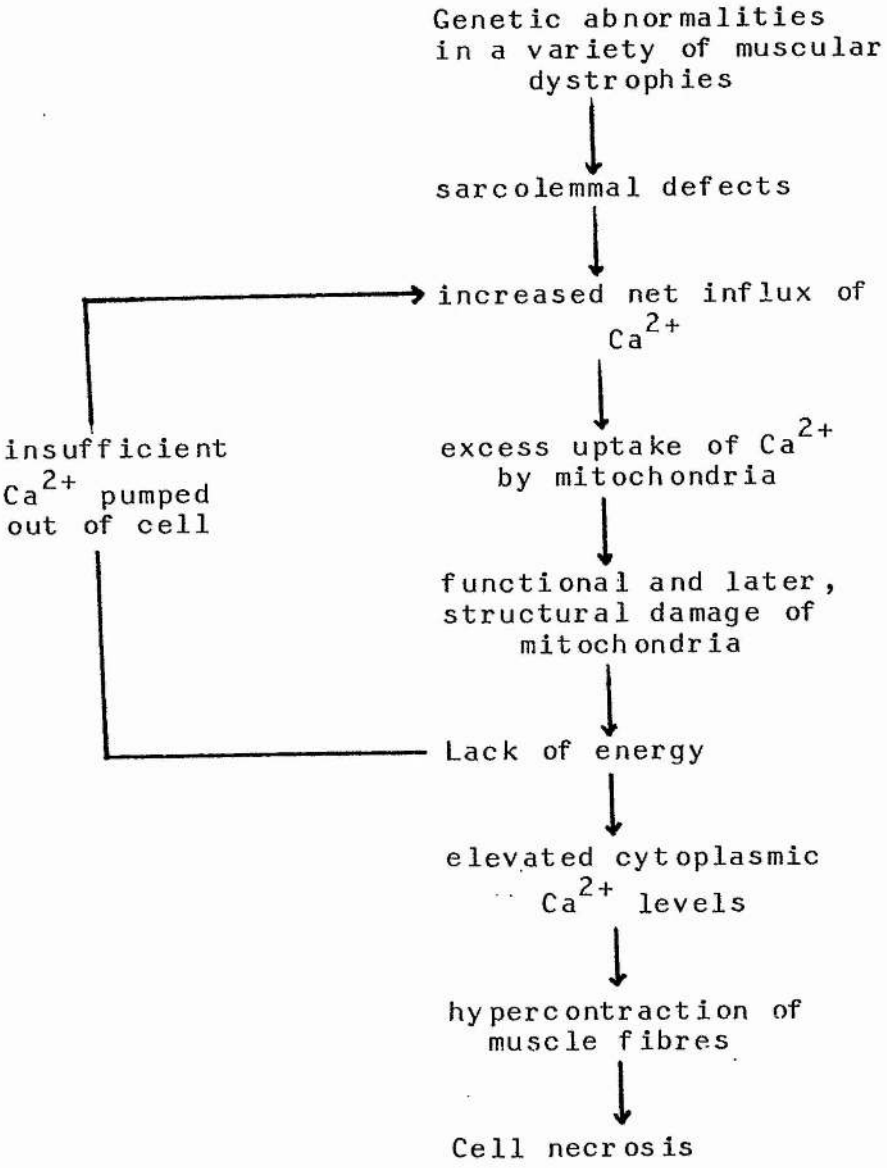
The membrane theory proposes that there is a systemic abnormality in the plasma membrane. This abnormality in the membrane, according to classical genetic theory, should be manifest in all tissues, even though it is apparently expressed as a clinical defect in muscle. Roses (1977) has suggested that 'whether or not a particular tissue presents clinical symptoms or signs may depend on the relative importance of the specific biochemical defect for the function of that tissue'.

Muscle necrosis and the serum changes can be explained by the Membrane theory. Duncan (1978) proposed that in DMD, the significant genetic defect is in the Ca^{2+}-channels of the muscle plasma membrane so that Ca^{2+}-influx is increased. Ca^{2+}-uptake by sarcoplasmic reticulum (SR) may also be impaired. As a consequence Ca^{2+} rises in the sarcoplasm and the muscle attempts to correct the position by storage of Ca^{2+} in the nucleus and mitochondria. Ca^{2+} is released from the SR in excitation and, in DMD muscle, Ca^{2+} may then be raised to a level sufficient to promote a Ca^{2+}-induced release of Ca^{2+} from the SR due to a positive feed-back mechanism, so increasing Ca^{2+} to a concentration which triggers protease activity directly or indirectly, or promotes the release of lysosomal enzymes, which leads to fibre necrosis.

Similarly, Wrogemann and Pena (1976) proposed a sarcolemmal defect increased the net influx of Ca^{2+} into cells which triggers a 'vicious cycle of mitochondrial,

calcium overloading, energy depletion and then cell necrosis'.

The hypothesis can be summarized by the following diagram:



Bradley and Fulthorpe (1978) studied the integrity of sarcolemma with the dye Procion Yellow. The dye entered fibres more often than in control fibres. They also suggested that sarcolemmal defects may allow entry of a high concentration of Ca^{2+} into the sarcoplasm with consequent fibre damage due to activation of proteinases by Ca^{2+}.

A physical interruption of the muscle cell or surface membrane has been proposed as the cause of the increased serum enzyme activities in patients with DMD, but the story seems more complicated. Two lines of evidence support this assumption.

1. Increased serum enzyme activities can be measured before there is actual muscle necrosis (Carpenter and Karpati, 1979).
2. There does not seem to be strict proportionality between the number of necrotic fibres and the magnitude of the serum enzyme abnormality in either DMD patients or carriers (Roy and Dubowity, 1970).

In short, the evidence implies that a genetic abnormality of surface membrane may be the fundamental cause of the high serum enzyme activity in DMD (Rowland, 1980).

Most of the research work in the muscular dystrophy field is directed towards cells other than muscle cells. The practical problems in obtaining muscle biopsies and pure sarcolemma free of contamination from connective

tissue (which is more abundant in DMD muscle) and the relatively poor state of knowledge about the structure and function of the normal sarcolemma and sarcoplasmic reticulum, have directed most of the research work towards red blood cells (RBC) and fibroblasts. Fibroblasts are attractive as experimental material because of the relative ease of culture in vitro, but most investigations on non-muscle cells have been with RBC. RBC provide an easily accessible, repeatable, controlable source of material for experiments (Roses, 1977). Much of our knowledge of normal membrane structure and function has been obtained from studies on these cells. Some workers consider this approach to be mistaken because the RBC is a highly specialized cell that is not only different in structure from muscle cells but also is unable to perform many normal biochemical functions, e.g. protein synthesis, oxidative phosphorylation, since it lacks subcellular organelles (Lucy, 1980). Roses (1977) also argues that muscle cells in culture from patients may no longer demonstrate the biochemical defect since they are a product of the dystrophic process and may represent a selective population consisting only of healthy regenerating cells.

Irrespective of the relative merits of using cells other than muscle cells in studying the primary defect of DMD, much evidence to support the Membrane Theory has been found in muscle (both biopsied and in culture), RBC, cultured skin fibroblasts, lymphocytes

and other somatic tissue. The following sections will present the evidence found in recent years.

1.4 Evidence to Support Membrane Theory

1.4.1 Membrane Lipids

1.4.1.1 RBC

Many contradictory results have been obtained for analysis of the phospholipids, neutral lipids and their fatty acids by different groups of workers.

Content of cholesterol and total phospholipids as well as phospholipid composition were found to be normal in RBC in both patients and carriers. (Kobayashi, Mawateri and Kuroiwa, 1978; Koski, Jungalwala and Kolodny, 1978; McLaughlin and Engle, 1979; Godin, Bridges and MacLeod, 1978). In contrast, Kalofoutis, Jullien and Spanos (1977) found a decrease in phosphatidyl-choline (PC), but increases in sphingomyelin (SM) and lyso-phosphatidylcholine (LPC).

Some workers found a normal lipid class composition in RBC membrane but altered fatty acids components.

An increase in stearic acid ($C_{18:0}$) and decrease in behenic acid ($C_{20:0}$) were found in the LPC fraction of DMD RBC (Ruitenbeek, 1978). Three reports described a decrease of palmitoleic acid: in diglyceride and triglyceride (Ruitenbeek, 1978; Howland and Iyer, 1977) and in SM and phosphatidyl ethanolamine (PE) (Beyer, Bieth, Freysz, Robert and Uhl, 1981). An increase in arachidonic acid ($C_{20:4}$) in SM fraction was also found in the RBC of patients and carriers (Beyer *et al.*, 1981).

Kunze, Reichmann, Egger, Leuschner and Eckhardt (1973) showed an increase in SM in RBC membrane and a

normal content of plasmalogens. An increased content of dimethyl acetals and a decrease in arachidonic acid ($C_{20:4}$) were found in PE. An increased percentage of stearic acid ($C_{18:0}$), but decreased palmitic acid ($C_{16:0}$), tetracosadienoic acid ($C_{24:2}$) and linoleic acid ($C_{18:2}$) were found in SM. This report and that of Kalofoutis et al. (1977) were critisized by most of the subsequent workers. The relative percentages of SM, PC, PE and phosphatidylserine (PS) of normal RBC obtained by Kunze et al. were different from the normal accepted figures (PC. 34.6%, SM 30.7%, PE + PS 34.6% compared to PC 29%, SM 25-27% and PE + PS 42%).

Some workers argued that the diminished content of the relatively more unsaturated components (PS and PE) and the increased percentage of SM obtained by Kunze et al. and Kalofoutis was due to the lack of inclusion of antioxidant in extraction and chromatography solvents and other precautions against lipid peroxidation during the experiment. Dodge and Phillips (1966) indicated that autoxidation of lipids may lead to loss of the more unsaturated species, PE and PS, and to a lesser extent PC. The increase in SM reported by these two groups in DMD may, therefore, be artifactual and due to the preferential loss of the more unsaturated glycerophospholipids. However, if the samples in their studies were all treated identically (especially length of time between sampling and analysis) the fact that a difference was found between patients and controls may infer significantly increased susceptibility of lipids to

peroxidation (Hunter, Brzeski and de Vane, 1981).

In summary, six reports of a normal phospholipid distribution were presented compared with only two laboratories which found abnormal phospholipid composition. Now it is generally accepted that, in the RBC membrane, no abnormality of major phospholipid composition exists. The highly variable and sometimes conflicting data concerning fatty acid composition need further confirmation before any conclusions can be drawn. Nevertheless, it is still possible that there is an abnormality in some quantitatively minor but functionally important lipid components, e.g. glycolipids and phosphoinositides.

1.4.1.2 Muscle

1.4.1.2.1 Muscle biopsies

Niebrój-Dobosz (1980) was able to show an increase in total concentration of phospholipid in total DMD muscle plasma membrane.

The SM content of DMD muscle biopsies was reported to be increased (Kunze and Olthoff, 1970; Hughes, 1972; Takagi, 1971; Pearce, Johnsen, Wysocki and Kokulas, 1981) whilst that of PC was found to be decreased (Kunze et al., 1970; Hughes and Takagi, 1971). The lipid components were also reported to be changed and different causes were proposed for these alterations.

Kunze et al. (1970) suggested the decreased PE and increased LPC were the consequence of an enzyme defect in the disease. A similarity between immature muscle and DMD muscle was proposed by Hughes (1972) as he found

an increase in total cholesterol and a decrease in choline plasmalogens in DMD muscle biopsies. Pearce et al., (1981) attributed the changes they found in DMD muscle biopsies to the increased amounts of fat which infiltrate the dystrophic muscle since the variation observed was found to parallel the degree of infiltration of muscle with fat and connective tissue as observed microscopically.

Conflicting data exist regarding the fatty acid composition of dystrophic muscle. Susheela (1968) reported a normal free fatty acid concentration in muscle of DMD patients. Kunze, Reichman, Egger, Olthoff and Döhler (1975) and Takagi, Muto, Takahashi and Nakas (1968) showed a normal fatty acid pattern in triglycerides, SM and PE of DMD muscle, but noted changes in the PC fraction. A decrease in linoleic acid ($C_{18:2}$) and a corresponding increase in oleic acid ($C_{18:1}$) were found. They showed that the fatty acid pattern of PC of DMD muscle was different from that of neonatal muscle, in contrast to Hughes (1972) suggestion that their lipid compositions are similar.

The lipid components of sarcoplasmic reticulum of DMD muscle were reported to be changed by Takagi, Schotland and Rowland (1973) who found an increase of SM and a decrease in PC and phosphatidyl inositol (PI). However, they suggested that it was due to contaminating tissue.

Surveying the reports of the phospholipid content of dystrophic muscle, a decrease in PC and increased SM

are the most consistent results obtained by different groups of workers. However the possibility still remains that the muscle studied was contaminated by the infiltrated adipose and connective tissue as suggested by Pearce et al. (1981).

1.4.1.2.2 Cultured Muscle

Bonsett, Rudman and Elliott (1979) studied DMD muscle explants and showed an increased number of fat droplets compared with controls under identical tissue culture condition. They suggested an abnormality in fat metabolism in DMD skeletal muscle and/or other cell types.

1.4.1.3 Cultured Skin Fibroblasts

No abnormality of the phospholipid profiles and fatty acid composition of DMD cultured skin fibroblasts was found in either young or old cultures (Kohlschütter, Wiesmann, Herschkowitz and Ferber, 1976). This is the only published report on lipid composition in this cell type although preliminary studies in our laboratory (Hunter, unpublished observations) confirm these findings.

Overall, the reports of the lipid and fatty acid compositions of dystrophic RBC, muscle (biopsies and cultured explants) and cultured skin fibroblasts, show no abnormalities in the major phospholipid classes. Although some changes were found in dystrophic muscle biopsies, it was strongly suspected to be contamination from infiltrated fat and connective tissue. However, few studies have been performed to investigate the minor lipid components which may be functionally important, this may be a profitable field of further study.

1.4.2 Membrane Proteins

1.4.2.1 Spectrin

The erythrocyte membrane 'cytoskeleton' is a two-dimensional network of proteins located at the inner (cytoplasmic) surface of the red cell membrane. The major proteins of this network are spectrin (bands 1 and 2 in the SDS electrophorogram) and actin (band 5). Since spectrin constitutes approximately 60-70% of the cytoskeleton proteins, it is likely that these structures represent fibres of the cytoskeleton (Pálek and Liu, 1980). Spectrin is likely to be involved in determining the shape of RBC, in stabilizing the lipid bilayer and may also contribute to some of the lipid asymmetry of the red cell membrane (Marchesi, 1979). The state of spectrin phosphorylation may also influence the shape of RBC (this will be discussed in more detail in the following section), the asymmetrical arrangement of the lipid bilayer and, in consequence, the activities of some membrane bound enzymes (Marchesi, 1979).

Missirlis (1978) and Vickers, McComas, Kohn and Rathbone (1978) were able to show the rates of phosphorylation of spectrin and band 3 protein were significantly higher in ghosts prepared from DMD patients. Rates for both spectrin and band 3 were altered to the same extent and they suggested a common cause, possibly the lipid environment. An increase in ^{32}P incorporation into membrane protein in DMD carriers was indicated (Tagliavini, Grassi, Rossi and Conterio, 1980). Mabry and Roses (1981) found an increase in phosphorylation of tryptic peptides of spectrin II in DMD RBC. The enzyme which is res-

ponsible for the phosphorylation of spectrin is the protein kinase which was found to be increased by Missirlis et al. (1978).

The phosphorylation of Band II has been studied in detail by Roses et al. (1975; 1976; 1977; 1979; 1981) and was found to be increased. They indicated a sensitive interrelationship between Ca^{2+} and the increased phosphorylation of Band II and suggested Ca^{2+} may play a role in maintaining membrane integrity.

In another study by Roses and Appel, (1976) the in vivo phosphorylated state of spectrin in the membrane was found to be the same in DMD and controls RBC. Furthermore the kinetic evaluation of protein kinase demonstrated no difference in DMD. They suggested an abnormal substrate or alteration in the state of the vectorial and geometrical relationship of substrate to enzyme due to other membrane factors or constitutents. This might therefore explain the discrepancy between in vitro and in vivo findings. The individual segments of spectrin have variable efficiencies as substrate for the endogenous protein kinase. Roses et al. (1981) tried to identify the abnormal substrate in Band II and found more than 50% of the ^{32}P was associated with a single cyanogen bromide cleavage band only (CN-A), although the CN-A band only represents about 2% of the total peptide protein.

Roses (1977) related the significance of increased phosphorylation of band II to the pathogenesis of DMD. The amino acid composition of spectrin is similar to

muscle moysin. He proposed that an abnormal type or polymorphic form of myosin may be present in DMD. This abnormality might affect the exquisite 3-dimensional structure of the myofibrillar elements resulting in dissolution, dystrophy and multiple secondary effects.

Roses' conclusion needs further confirmation since contradictory results from several laboratories have been presented. Tortolero, Fischer, Pian, Delaunay and Schapira (1979); Iyer, Hoenig, Sherblom and Howland (1977); Fischer, Tortolero, Delaunay and Schapira (1978); Falk, Campion, Guthrie, Sparkes and Fox (1979) found no difference in Band II phosphorylation but agreed with Roses that the protein kinase activity was unchanged (Tortolero et al., 1979).

In addition to the controversial data on spectrin phosphorylation, Tsuchiya, Sugita, Ishiura and Imahori (1981) and Nagano, Wong and Roses (1980) indicated spectrin extractability was significantly reduced in DMD ghosts and suggested that spectrin from DMD patients was more tightly bound to the membranes.

In a critical review by Tsung and Palek (1980) conflicting results for RBC membrane protein phosphorylation were related to differences in methods including ghost-preparation and enzyme-assay conditions. They suspected the data of Roses et al. to be difficult to interpret, due to extremely low ^{32}P per mole of spectrin, that is, only about 40% of the total number of exchangeable phosphorus atoms per mole of spectrin.

Due to the close resemblance between RBC spectrin and muscle myosin, the importance and significance of the phosphorylated state of band II can not be ignored and needs further investigation.

1.4.2.2 Freeze-Fracture Studies

Using the freeze-fracture electron microscope technique, Wakayama, Hodson, Pleasure, Bonilla and Schotland (1978) and Wakayama, Schotland, Bonilla and Orecchio (1979) showed a decrease in number and uniform distribution of intramembranous particles (P phase and E phase) in DMD RBC membrane. They suggested this was the consequence of a change in properties of band 3 peptides or in the membrane lipid with which they interact to form the intramembranous particles.

In DMD muscle, non-uniform distribution of intramembranous particles including orthogonal arrays were found on both protoplasmic and extracellular faces of the plasma membrane (Schotland, Bonilla and Meter, 1977; Schotland, Bonilla and Wakayama, 1980; 1981). In contrast, Osame, Engel, Rebouche and Scott (1980) found a normal frequency and distribution of intramembranous particles in cultured DMD muscle fibres.

Study of membranous protein distribution by observing concanavalin A binding under E.M. showed normal, regular and continuous binding sites in cultured DMD muscle (Heiman-Patterson, 1982). But Bonilla, Schotland and Wakayama (1978) found the DMD muscle fibres (plasma membrane and basal lamina) showed irregular and patchy

concanavalin binding sites. They suggested the focal abnormal regions of Con A binding sites were associated with a focal increase in extracellular collagen, and alterations in distribution of α-D-mannose, α-D-glucose and α-D-fructose.

The negative results both came from studying cultured muscle fibres in contrast to the positive results obtained by using DMD muscle biopsies. The difference between DMD fresh muscle fibres and cultured muscle fibres is unknown, but it may be that only healthy muscle cells will establish themselves in culture (Roses, 1977).

1.4.2.3 Cultured Skin Fibroblasts

Jones and Witkowski (1981) measured the aggregation of cultured fibroblasts in a Couette viscometer. Results showed the DMD cells have a low intercellular adhesiveness and formed small numbers of very large aggregates which were absent in normal cells. They suggested these might be an alteration in the surface of dystrophic cells. But the mean particle densities and coefficients of dispersion of the intramembranous particles of DMD cultured skin fibroblasts showed no difference from that of normal cells (Jones, Severs and Witkowski, 1981). One might expect the abnormal surface characters of the DMD cultured fibroblasts to reflect some abnormality in their membrane proteins and/or lipids especially glycoproteins or glycolipids. A recent study (Burghes, Dunn, Statham and Dubowitz, 1982) of DMD cultured skin fibroblasts protein by SDS-gel electrophoresis showed

the regions of 73,000-68,000 and 48,000 MW were decreased while those of 175,000 and 53,000 MW were significantly elevated. A normally functioning cytoskeleton was observed by staining with concanavalin in DMD skin cultured fibroblasts (Newman, 1982). Pena, Vust, Tucker, Hamenton and Wrogemann (1978) also demonstrated a normal distribution of subcellular fractions and normal major protein components.

Evidence from DMD RBC spectrin phosphorylation, freeze-fracture E.M. of muscle and RBC, and electrophoretic analysis of cultured skin fibroblasts favors an abnormality in membrane protein, which might explain most of the abnormalities found so far. However, the evidence relating to spectrin is equivocal and isolation of an abnormal protein from muscle or fibroblasts has not been achieved and this area needs further investigation to elucidate this problem.

1.4.3 Physical Properties of Membranes

1.4.3.1 Shape and Deformability of RBC

The deformability of the RBC depends essentially on three factors (Mohandas, Phillips and Bessis, 1979).

1. The state of the cell contents i.e. the internal viscosity of the intracellular haemoglobin milieu, as regulated by the mean cell haemoglobin concentration and the state of haemoglobin aggregation.
2. The geometry of the cell i.e. the overall shape and the surface area to volume relationships.
3. The membrane properties i.e. the deformability, elasticity, viscosity and fragility of the membrane.

The membrane deformability, flexibility and morphology (shape) are primarly dependent on, and controlled by, a sub-membranous cytoskeleton (Lux, 1979; Marchesi, 1979; Palek and Liu, 1980). The phosphorylated state of spectrin will markedly influence the shape and deformability of the RBC membranes (Birchmeier and Singer, 1977). Thus the deformability and shape of RBC may reflect the intrinsic biochemical state of the membrane. Shear Modulus (dyne/cm) is a measure of the viscoelastic properties of the membrane. Increase in Shear Modulus, i.e. increase resistance to deformation were found in DMD RBC (Brain, Kohn, McComas, Missirlis, Rathbone and Vickers, 1978; Missirlis and Kohn, 1978; Kohn, Missirlis, McComas and Brain, 1977). But Somer, Chieu Sung and Thuru (1979) demonstrated no significant difference in membrane deformability determined by microsieving and flow channel measurements. A normal haemoglobin content, viscosity and flexibility of DMD RBC membrane, which reflects a normal deformability has also been indicated (Tillman, Lenard, Wagner, Dŏngs and Shröter, 1979).

An increased percentage of echinocytes (distorted RBC) in DMD blood was found by Matheson and Howland (1974); Korczyn, Fishman, Djaldetti and Berginer (1977); Lumb and Emergy (1975); Grassi (1978); Beyer, Stephan, Oberling and Daya (1977); Howells (1976); Percy and Miller (1975); Miller, Roses and Appel (1975). Both Percy and Miller (1975) and Howells (1976) suggested an abnormality in spectrin and actin might be responsible. Conflicting reports of a normal percentage of echinocytes

in DMD blood have also appeared (Szentistványi and Janka (1977); Miale (1975); Soltan (1977); and Mollica (1980)) and this normality is also seen after glutaraldehyde fixation under phase contrast and scanning E.M. (Szentistványi and Janka, 1977). Matheson (1976) retracted his finding of changed RBC morphology (Matheson, 1974) after using various methods to prepare erythrocyte samples for observing morphology and found no significant abnormality of RBC shapes. He concluded that RBC shape was very sensitive to various type of cell treatment and might be the cause of the controversial reports about DMD RBC morphology.

At the present time, there is still no unchallenged finding regarding the deformability and morphology of DMD RBC. However a membrane defect (perhaps of spectrin) may exist and be reflected by the different responses of DMD and normal cells to various cell treatments.

1.4.3.2 Osmotic Fragility of RBC

Osmotic fragility of RBC is governed by the excess surface area to volume ratio (i.e. degree of swelling) and by the elastic properties of the membrane which enable it to undergo these deformations without lysis (Weed and Reed, 1966). The rate of lysis is determined by the degree of rupturing of the lipid bilayer, which occurs after the cells have become spherical. Osmotic fragility of the cellular membrane is directly proportional to the degree of fluidity of the lipid bilayer. It seems that the spectrin network and degree of membrane

deformability are not the rate determining factors of haemolysis (Araki and Rifkind, 1981). Any changes in degree of fragility therefore, may reflect a fluidity change in the membrane.

Danieli and Marchesini (1980) showed an increased RBC osmotic fragility in both DMD patients and carriers and this phenomenom was enhanced by ATP depletion. This result (in DMD patients) was confirmed by Lloyd and Nunn, (1978); Fisher, Silvestri, Vester, Nolan, Ahmad and Danowski (1976); Kim, Luthra, Watts and Stern (1980). But Ruitenbeek, Edixhoven and Scholte (1979) found that after phospholipase (EC 2.1.1.4) treatment (from pancreas snake venom or bee venom) there was no difference between the osmotic fragility of DMD and control RBC, both being increased by the same amount. Theyconcluded that phospholipid packing was not much altered in DMD patients. A more direct way to measure phospholipid packing and fluidity is by using various kinds of probes and spectroscopic techniques, particularly ESR, NMR and fluorescence.

1.4.3.3 Spectroscopic Studies

Biophysical probe techniques yield detailed information on molecular organization. The techniques commonly used are electron spin resonance spectroscopy (ESR), fluorescent techniques and nuclear magnetic resonance spectroscopy (NMR). Using ESR and lipid soluble spin probes with different chain lengths it is possible to measure the fluidity at different depths of the lipid

bilayer. Fluidity is a consequence of the degree of unsaturation, chain length of the fatty acyl groups and hence the phospholipid packing of the lipid bialyer. Probes can also give information about conformation of the intrinsic protein, membrane polarity and protein-lipid interactions. Any change in lipid bilayer physical properties may affect the conformation of intrinsic proteins and, in consequence, membrane functions.

Many conflicting results have been presented concerning the lipid fluidity of the polar (surface) region, hydrophobic (non-polar) core and the protein conformation of the DMD RBC. They are summarized in the following three tables (Tables 3, 4, and 5).

Different results may depend on the type of spin label used or the type of ESR. The report of Wilkerson, Perkins, Roelofs, Swift, Dalton and Park (1978) is noteworthy because they used the same kind of probes (5-NS) as that of Butterfield, Chesnut, Appel and Roses (1976); Butterfield (1977); Butterfield and Markesbery (1980) and Laurent, Daveloose, Leterrier, Fischer and Schapira (1980), but studied with the specialised technique ST-EPR. ST-EPR can study motions of macromolecules and slower molecular reorientations as slow as 10^{-3} sec, while the lower limit of conventional ESR is 10^{-7} sec. It seems that the results (decreased fluidity in polar region of DMD ghost) of Wilkerson et al. (1978) is therefore more reliable than previous findings. A different method (fluorescence polarization) and different probes used (1,6-diphenyl 1,3,5-hexatriene) by Dunn (1980) also gave a similar result to that of Wilkerson et al. (1978).

Fluidity	Probes used	Techniques	Remarks	Refs.
Normal	I(1.14)	ESR		Sato, Nishikida, Samuels and Tyler, 1978
Normal	5-NS	ESR		Butterfield et al., 1976; 1977; 1980
Normal	5-NS	ESR		Laurent et al., 1980
Decreased	1,6-diphenyl, 1,3,5-hexatriene	fluorescence polarization	most apparent at less than 22°C	Dunn, 1980
Decreased	5-NS	saturation transfer electron paramagnetic resonance	measures slower motions than ESR	Wilkerson et al., 1978
Altered	lipid-soluble spin labels	ESR	depletion of extrinsic proteins and intramembranous particles?	Falcioni, Ferretti, Mazzanti and Caratola, 1982
Increased	5-NS	ESR	alterations in lipid-protein organisation?	Dellantonio, Angeleri, Capriotti, Lenaz, Curatola, Mazzanti and Bertoli, 1980

Table 3

Lipid fluidity at the surface (polar) region of DMD ghost.

Fluidity	Probes used	Techniques	Remarks	Refs.
Increase	I(1.14)	ESR	different protein-lipid interactions?	Sato et al., 1978
Altered	lipid-soluble spin labels	ESR	previous table	Falcioni et al., 1982
Increased	16-NS	ESR	Structural alteration of the phospho-lipid organ-isation of DMD RBC membrane?	Laurent et al., 1980
Altered	16-NS	ESR	abnormal lipid domain?	Leterrier, Daveloose, Laurent, Fischer and Schapira, 1980
Normal	C-12	ESR	Measured at 7-50°C on whole RBC	Branca, Gamba, Mastropado, Serra, and Sulas, 1980
Normal	16-NS	ESR		Dellantonio et al., 1980

Table 4

Lipid fluidity at the hydrophobic region of DMD ghost

In addition, Sarpel, Lubansky, Danon and Omachi (1981) measured the lipid profile (especially PC) on whole RBC of DMD patient by ^{31}P NMR, and found a normal lipid feature.

Conformation	Probes	Technique	Remarks	Refs.
Altered	MAL-6	ESR		Butterfield et al., 1976; 1977; 1980
Altered	MAL-6	ESR	Altered membrane architecture?	Dellantonio et al., 1980
Normal	maleimide nitroxide III	ESR		Laurent et al., 1980
Normal	MAL-6	ESR	Measured at 7-50°C on whole cell	Branca et al., 1980

Table 5 Protein Conformation of the DMD Ghost

Footnotes of the Tables:

I(1.14) 2-(14-carboxytetradecyl)-2-ethyl-4,4-dimethyl-3-oxazol-idinyloxyl

5 NS 2-(3-carboxypropyl)-2-tridecyl-4,4-dimethyl-3-oxazolindinyl-oxyl; (5 nitroxide stearic acid)

MAL 6 2,2,6,6-tetramethylpiperidin-oxyl-4-maleimide

16 NS 16-nitroxide stearic acid

C 12 4-hydroxy-2,2,6,6-tetramethylpiperidinooxyl dodecane

Polarity was found to be increased by Statham, Dunn, Rice and Evans (1981) and Scarlato, Merla, Silani, Manfredi, Battiroli and Zanelk (1979) using a fluorescent probe.

The lipid fluidity of the hydrophobic region of DMD RBC seems to be altered since four reports indicated this using two different kinds of probes, and only two reports gave a negative result.

A change in lipid profile may influence membrane protein conformation and may be reflected in the altered protein conformation suggested by Dellantonio et al. (1980) and Butterfield et al. (1976; 1977; 1980).

Although the physical techniques mentioned above enable the observation of the intramembraneous micro-environment, the limitations of these techniques can not be ignored. Many spin-label reporter groups and fluor-escent molecules are rather bulky. When attached to normal membrane constituents such as phospholipids or fatty acids, they may cause local perturbations of membrane structure. Thus the probe molecules may actually report the physical properties of an unnatural micro-environment which they create themselves, rather than accurately indicating the physical properties of the membrane itself. This disadvantage can be minimized by the synthesis and ultilization of spin-label or fluorescent probes which more closely resemble native membrane constituents. The location of the probes may not be as designed, but depend on the depth of penetration and problems resulting from lateral phase separation. A change in fluorescence intensity of probe molecules may indicate an alteration in its environment polarity, but it may also be due to static or dynamic quenching, absorption effects, energy transfer and solvent

immobilization. Failure to consider such problems may lead to erroneous conclusions (Barchi, 1980). But even if some biophysical data is artefactual, when the control and DMD cells are treated identically a different result obtained between DMD and normal cells may still mean that there is an essential difference in membrane properties.

Although a normal phospholipid distribution is found in DMD RBC, a change in lipid packing, for example, a change in the asymmetrical distribution of the phospholipid, could influence the fluidity of the membrane which may be reflected in a change in deformability, osmotic fragility and physical state of the RBC membrane. The results presented so far seem to favor a change in the physical state of the DMD RBC membrane.

1.4.4 Ion Transport and ATPases

Any change in the overall physical state of the membrane may affect either passive or active ion transport. All ion transport enzymes (pumps) are membrane-bound, and changes in their activities may reflect the abnormalities occuring in the membrane.

1.4.4.1 Ion Transport

1.4.4.1.1 Ion Transport in RBC

Dise, Goodman, Lake, Hodson and Rasimussen (1977) were able to demonstrate an increase in K^+ efflux from RBC of DMD patients and proposed that this phenomenon might be due to increased sensitivity to Ca^{2+}. Converse findings were obtained by Szentistványi, Janka and Heiner (1980) who found a significant reduction in Ca^{2+}-dependent K^+ efflux and suggested these changes might be due to

alterations in Ca^{2+} homeostasis and/or in the mechanism of active and passive Ca^{2+} transport of intact red cells. Mollman, Cardenas and Pleasure (1979; 1980) prepared inside-out vesicles and examined the mean K_m and V_{max} (for Ca^{2+}) of the Ca^{2+} transport protein (ATPase). Results showed a significantly increased K_m and V_{max} in RBC of patients with DMD, i.e. a decreased affinity for Ca^{2+} but transport of Ca^{2+} at a faster rate. They suggested this might be due to an abnormal interaction between the ion-transport activator and the transport system rather than to a primary defect in transport system itself. But a later report (Shoji, 1981) found no significant difference in Ca^{2+} influx and efflux in DMD and control RBC.

Because of the possible important role of Ca^{2+} in activating the proteolytic enzymes, which may be the cause of the degeneration of muscle fibres, Ca^{2+} has received much attention. Conflicting results concerning Ca^{2+} transport were presented, so some research workers examined Ca^{++}-ATPase rather than ion transport itself in RBC (see section 1.4.4.2.1).

1.4.4.1.2 Ion Transport in DMD Muscle

The kinetics of Ca^{2+} accumulation by isolated sarcotubular vesicles was found to be impaired (Takagi, Schotland and Rowland, 1973; Wood, Sorenson, Eastwood, Charash and Reuben, 1978), as demonstrated by a significant decrease in the ability of isolated vesicles to accumulate Ca^{2+}. This conflicted with the finding of Peter (1969)

that the capacity of vesicles to accumulate Ca^{2+}, in excess Ca^{2+}, was normal.

An increase in Ca^{2+} in DMD skeletal muscle was found by Ca^{2+}-staining in DMD muscle biopsies (Bodensteiner and Engel, 1978). They suggested that the Ca^{2+} was intracellular, and might be due to alteration of membrane permeability or structural defects in sarcolemma. An increase in eosinophilic fibres (i.e. containing Ca^{2+}) was found in fetal DMD muscle (Emery and Burt, 1980), which suggested that it was an early biochemical change in the disorder. Since increased intracellular Ca^{2+} would account for various biochemical and clinical features of the disease, it might be related to the primary defect.

The least conflicting and contradictory results discussed so far concern Ca^{2+} accumulation in muscle.

1.4.4.2 ATPase

1.4.4.2.1 (Na^+K^+)-ATPase in DMD RBC

(Na^+K^+)-ATPase (Na^+-Pump) in erythrocytes has been extensively studied by many laboratories and controversial results obtained. The effect of ouabain on DMD (Na^+K^+)-ATPase has received much attention.

A change in the response of the enzyme to an inhibitor (or effector) may reflect a change in the shape of the allosteric or active sites, i.e. a conformational change. Since the lipid domain of the membrane is important in affecting the conformation of membrane-

bound enzymes, study of the response of the enzyme to its inhibitor may indicate abnormalities in the lipid domain.

Arrhenius plots of membrane-bound enzymes can demonstrate the lipid composition and arrangement in the immediate vicinity of the enzyme, since in many cases discontinuities in Arrhenius plots of membrane-bound enzyme or transport functions reflect lateral phase separations and phase transitions occuring in the lipid environment surrounding the protein.

The Arrhenius plot of the (Na^+K^+)-ATPase of normal ghosts is biphasic and has a break at 21°C. The Arrhenius plot of DMD ghosts was found to be normal by Dunn, Burghes and Dubowitz (1982) but abnormal (linear) by Austin, Kwok, Erickson, May and Jeffrey (1979) and Austin, Kwok, May and Jeffrey (1980), suggesting a more fluid environment for the enzymes in DMD cells.

In normal RBC ghosts (Na^+K^+)-ATPase is inhibited by the cardiac glycoside, ouabain, which binds to the extracytoplasmically exposed protein of the pump. Conflicting results about the effect of ouabain on (Na^+K^+)-ATPase of DMD ghosts have been presented and probably constitute the most controversial experimental findings so far in relation to the disease. These are summarized in the following Table (Table 6).

The conflicting results for DMD ghost (Na^+K^+)-ATPase activity and its response to ouabain may be due to differences in ghost preparation, the ion content and composition of the buffer, time of incubation etc. Some

Basal Activity	Response of ATPase activity to 10^{-4}M ouabain	Remarks	References
Decreased	stimulated	K^+ influx, Na^+ efflux were inhibited by ouabain	Pearson, 1978
Decreased	stimulated	Stimulated in both low sodium and high sodium (optimal to the enzyme) system	Niebroj-Dobosz, 1976
	stimulated		Guidic, 1980; Brown, Chattopadhyay, 1967; Araki, Mawatari, 1971.
	Either no inhibition or stimulation		Galbraith, Watts, 1980
Normal	inhibited but less sensitive		Mawatari, Shiro, Olarte, Schonberg, 1976
	stimulated or less sensitive	also shown in normal RBC if incubated with DMD serum prior to isolation of ghost or before assay. Peter et al., (1969) suggested some factor(s) in DMD blood might be responsible. This might be an autoallergic process in which damaged muscle membranes induced an antibody to membranes in general	Peter, Worsfold, Pearson, 1969; Siddiqui, Pennington, 1977; Lloyd, Emery, 1980

Basal Activity	Response of ATPase activity to 10^{-4}M ouabain	Remarks	References
Normal	inhibited		Klassen, Blostein, 1969; Hodson, Pleasure, 1977; Wacholtz, Raible, Jackowski, Rodan, Sha'afi, Rodan, 1979; Ruitenbeek, 1979; Souweine, Bernard, Lasne, Lachanat, 1978; Dunn, Purghes, Dubowitz, 1982; Probstfield, Wang, From, 1972; Mawatari, Igisu, Kuroiwa, Miyoshino, 1981

Table 6 Activity of (Na^+K^+)-ATPase and its response to ouabain in DMD erythrocytes

workers omitted EDTA from their assay to completely eliminate Ca^{2+}, so that, the so-called $(Na^{+}K^{+})$-ATPase activity included the activity of $(Ca^{2+}Mg^{2+})$ ATPase which is insensitive to the effect of ouabain. However, not all discrepancies in results were due to obvious differences in procedure. Klassen and Blostein (1969) followed the same method as Brown et al. (1967) to measure the $(Na^{+}K^{+})$-ATPase activity and response to ouabain, and obtained the opposite result to Brown, i.e. inhibition of DMD ATPase. Mawatari et al. (1981) tried to solve this controversy by measuring the $(Na^{+}K^{+})$-ATPase activity under different experimental conditions. They found the type of anticoagulants, extent of washing of the RBC, whether mechanical or osmotic disruption of the cells was used, presence or absence of EDTA in the washing solution for the ghosts, intentional removal of the peripheral proteins of the membranes, addition of DMD plasma to the assay system and different temperatures of membrane storage caused no significant differences between DMD and control in $(Na^{+}K^{+})$-ATPase or in response of the enzyme to ouabain.

If the abnormal activity of $(Na^{+}K^{+})$-ATPase in DMD RBC exists, it might be due to a change in the lipid domain as proposed by Mawateri et al. (1976). PS might be a suitable candidate as Roelofsen and van Deenen (1973) indicated the $(Na^{+}K^{+})$-ATPase activity was strongly dependant on the presence of PS.

1.4.4.2.2 $(Ca^{2+}$-$Mg^{2+})$ATPase in DMD RBC

Dunn and Burghes (1980; 1981; 1982) demonstrated an increasedactivity of $(Ca^{2+}$-$Mg^{2+})$ATPase in DMD and carriers RBC, which confirmed the results of Luthra, Stern and Kim (1979). The V_{max} and activation energy of this enzyme were also elevated. They suggested a change in the lipid domain may be responsible. Luthra et al. (1979) indicated that this enzyme in DMD ghosts was stimulated by a cytoplasmic activator to the same extent in patients and controls so that the difference was related to a difference in membrane structure in DMD ghosts rather than to alterations in the activator protein. Another two reports also found increased activity of $(Ca^{2+}$-$Mg^{2+})$ATPase (Hodson and Pleasure, 1977; Ruitenbeek, 1979). Ruitenbeek (1978) proposed that calcium metabolism in RBC of DMD might be impaired as a consequence. Also he found two affinity sites were present in DMD $(Ca^{2+}$-$Mg^{2+})$ATPase while that of the controls only had one affinity site. Unlike many other reported abnormalities in DMD RBC, only one group has found a normal $(Ca^{2+}$-$Mg^{2+})$ATPase activity (Tsuchiya, Sugita, Ishuira and Imahori, 1981).

A study of the lipid requirement of $(Ca^{2+}$-$Mg^{2+})$ ATPase in human RBC by Roelofsen and Schatzmann (1977) suggested that only the glycerophospholipids are involved in the maintenance of the $(Ca^{2+}$-$Mg^{2+})$ATPase activity and in particular that fraction of these phospholipids located in the inner half of the membrane. An elevated

$(Ca^{2+}$-$Mg^{2+})$ATPase activity in DMD RBC may therefore be due to the changes in this lipid domain which is normally composed predominately of PS and PE with smaller amounts of PC and LPC.

Unlike the conflicting results relating to $(Na^{+}K^{+})$-ATPase, increased activity of $(Ca^{2+}$-$Mg^{2+})$ATPase has been confirmed by four groups and only one group reported a normal activity. An abnormal lipid (probably PS and PE at the inner side of the bilayer) could explain the generalized abnormal activities of $(Na^{+}K^{+})$-ATPase and $(Ca^{2+}$-$Mg^{2+})$ATPase. Further work is needed to confirm this hypothesis, especially to clarify the activity of $(Na^{+}K^{+})$-ATPase and its response to ouabain.

1.4.4.2.3 ATPases in DMD Muscle

Both $(Na^{+}K^{+})$-ATPase and $(Ca^{2+}$-$Mg^{2+})$ATPase in muscle biopsies and isolated sarcoplasmic reticulum have been investigated by several laboratories. The results are summarized in the following Table. (Table 7).

Totally conflicting results concerning ATPase activity in sarcolemma, sarcoplasmic reticulum and muscle biopsies have emerged making any firm conclusion impossible. The controversial results might be due to the practical difficulties of obtaining pure sarcolemma free of contaminating connective tissue and fat tissue which extensively infiltrates the DMD muscle. Also knowledge about membrane-bound enzymes in normal sarcolemma is not as complete as that in RBC. Further studies on the nature of normal sarcolemma may thus be

ATPase	Activity	Site	Remarks	References
(Na^+K^+) ATPase	decreased	sarcolemma	suggested a sarcolemmal defect	Dhalla, McNamara, Balaruleramanian, Greenlaw, Tucker, 1973
(Na^+K^+) ATPase	decreased stimulated by 10^{-4}M ouabain	muscle biopsies	suggested a change in activator/inhibitor, or disorganization of membrane structure probably a change in PS	Niebrej-Dobosz, 1981
(Na^+K^+) ATPase	increased (ouabain sensitive)	muscle biopsies		Sandhya, Das, 1982
$(Ca^{2+}$ $Mg^{2+})$ ATPase	increased	sarcolemma	suggested a sarcolemmal defect	Dhalla et al., 1973
$(Ca^{2+}$ $Mg^{2+})$ ATPase	decreased	muscle biopsies	suggested the cell may be unable to maintain high extracellular/intracellular Ca gradient, causing some inactivation of muscle fibre function	Niebroj-Dobosz, 1980
ATPases	decreased	sarcoplasmic reticulum	also found low initial rate, low total uptake of Ca^{2+}	Samaha, Gergely, 1969

Table 7 Activity of ATPases in DMD muscle

necessary before any conclusion about DMD muscle can be drawn.

1.4.5 Membrane-Related Enzymes in DMD

As a major type of gene product, altered activities of an enzyme(s) may reflect the primary genetic defect in DMD as is the case for a large number of other inherited diseases. Although no abnormal accumulation of any substrate, intermediate or end product has been found in the serum or urine of DMD patients, the primary defect may nevertheless be a metabolic one (example of enzyme deficiencies are known where the substrate for the defective enzymes does not accumulate). Alternatively the defect may be manifest as an alteration in non-enzyme protein (e.g. receptor or structural protein), or ion content of the tissue.

Understandably much effort in the field of DMD research has been directed to measuring enzyme activity, particularly of membrane-bound enzymes (including ATPases mentioned in the previous section).

1.4.5.1 Enzyme Changes in DMD RBC

The properties of the membrane lipid bilayer have received much attention due to its important role in the normal functioning of various membrane-bound enzymes. Many workers have proposed a change in the physical state of the lipid domain in DMD RBC, which has led to the study of enzymes involved in lipid metabolism.

Sherblom, McAllister, Macul and Howland (1980) studied the <u>in vitro</u> incorporation of ^{14}C-oleate into RBC membrane phospholipids. Results showed a diminished

incorporation and decreased Ca^{2+}-stimulation of fatty acid incorporation in RBC of patients with DMD compared with control. However, Kunze et al. (1973) reported that the in vitro influx of oleic acid ($C_{18:1}$) and its incorporation into PC and PE increased. Moore and Appel (1980) studied the methylation of PE to form successively N-monomethylphosphatidyl ethanolamine (PMME), N,N-Dimethyl-phosphatidylethanolamine (PDME) and PC but this was normal in RBC of DMD patients.

Iyer, Katyare and Howland (1976) demonstrated an increased phospholipase A activity in DMD RBC, whilst phospholipase C is reported to be 25% decreased (Austin, Kwok, Erickson, May and Jeffrey, 1979). The activation of long chain fatty acids by acyl-CoA synthetase and carnitine palmitoyltransferase activity in DMD RBC were unaltered (Ruitenbeek and Scholte, 1979). In their previous report in 1978, the same workers found palmitoleic acid ($C_{16:1}$) was decreased in triglyceride and diglyceride and so concluded that the change in palmitoleic acid was not due to impairment of the activation nor intra-mitochondrial transport of this fatty acid.

Lipid peroxidation, known to be involved in membrane damage in a large number of pathological states has also been proposed to underlie the membrane changes in DMD. Some enzymes responsible for protection from lipid peroxidation have been investigated but have given controversial results. Burri, Chan, Berry and Yarnell(1980)

indicated a 19% decrease of RBC superoxide dismutase (SOD) activity in patients with DMD, but both plasma SOD and plasma and RBC glutathione peroxidase were found to be normal. Matkovics, Laszlo and Szabo (1982) reported increased activities in SOD, catalase and lipid peroxidation in RBC of DMD patients. Hunter, Breski and de Vane (1981) and Hunter, Amin and de Vane (1983) showed normal activities of SOD catalase and glutathione peroxidase in RBC of patients with DMD. Their study did show a significant decrease in glutathione reductase activity although it was thought that this would not lead to increased peroxidation and membrane damage. Another study of unfractionated and age fractionated normal and DMD RBC showed normal glutathione reductase activity although this study included only two DMD patients (Galbraith and Watts, 1980). Adenylate cyclase, a membrane-bound enzyme was found to have decreased activity in DMD ghosts (Wacholtz, Raible, Jackowski, Rodan and Shaafi, 1979).

Relatively few studies of membrane related enzymes (particularly lipid-related) have been carried out and those that have been reported are largely controversial, once again, making firm conclusions difficult, but the report of Iyer et al. (1976) of an increased phospholipase A activity in DMD RBC is noteworthy since the product of phospholipase A (lysophosphatides) are well-known to be membrane disrupting agents.

1.4.5.2 Enzyme Changes in DMD Muscle

Some abnormalities have been found in lipid biosynthesis *in vitro* in DMD muscle. Kunze, Olthoff and Bönsch (1971) showed defective incorporation of linoleic acid by DMD muscle , using ^{14}C-labelling. They found decreased linoleic acid incorporation into SM, triglyceride and PC, and also decreased incorporation of palmitic acid into SM. A later study (Ionasescu, Monaco, Sandra, Ionasescu, Burmeister, Deprosse and Stern, 1981) showed a significant increase of ^{3}H glycerol incorporation into PC, PS and phosphatidylinositol (PI) and triglyceride in muscle biopsies and cultured muscle from DMD patients. As this incorporation pattern was similar to fetal muscle, they suggested the lipid alteration in dystrophic muscle might be related to a defect in maturation and lead to triglyceride accumulation. The similarity between fetal and dystrophic muscle had previously been pointed out by Hughes (1972) on the basis of lipid composition. Kunze and Olthoff (1969) studied the *in vitro* ^{32}P incorporation into phospholipid.

Results showed the specific activity of PC and LPC were especially increased. As PC was found to be decreased in DMD muscle, Kunze and Olthoff (1969) supposed increased turnover of PC might occur in DMD muscle. In a later report (1972) by the same authors, the acylation of LPC with stearic acid ($C_{18:0}$), palmitic acid ($C_{16:0}$) and linoleic acid ($C_{18:2}$) was reported to be normal,

i.e. acyltransferases were normal in biopsied muscle. The results of this work are highly doubtful however, because they used two females and one male with different forms of muscular dystrophies for experiment.

Chalovich (1979) demonstrated that muscle of DMD patients was deficient in glycerol-3-phosphorylcholine, and suggested this reflected changes in membrane metabolism through its precursors PC and LPC. Iyer et al. (1976) observed an increase phospholipase A activity in DMD RBC which implies possible accumulation of LPC. These phenomena prompted Schiselfeld, Barany, Danon, Abraham and Kleps (1981) to investigate LPC acyl hydrolase activity in DMD muscle. They found a normal maximal activity (V_{max}) but decreased affinity (raised K_m) for LPC. Kunze, Rüstow and Olthoff (1980) were able to show normal activities of CDP-choline: diglyceride-p-choline transferase, CDP-choline: ceramide-p-choline transferase and of PC degradation in DMD muscle. Berthillier (1982) showed increased activities of palmitoyl carnitine transferase and palmitoyl coenzyme A hydrolase but normal activity of palmitoyl coenzyme A synthetase in DMD muscle. Kar and Pearson (1975) indicated increased lipase activity (by 71%) in DMD muscles, and proposed the lipid accumulation in DMD muscle might be due to a defect in muscle lipases. In DMD muscle mitochondria, specific activities of the matrix enzyme, propionyl-CoA carboxylase, was found to be increased while palmitoyl-CoA synthetase activity was unchanged (Scholte and Busch, 1980). From

the decreased recovery of all outer membrane enzymesin the mitochondrial fraction they suggested an increased fragility of the outer mitochondrial membrane in DMD muscle and proposed mitochondria were involved early in the disease process.

Although the reports with respect to lipid metabolism are controversial, particularly concerning PC, on balance it is possible that the metabolism of PC might be altered. As a major phospholipid component in DMD muscle, any alteration in PC metabolism may give rise to a profound effect.

An increased level of lipid peroxidation in dystrophic muscle in which the lysosmal membrane is disrupted by the lipid peroxides, causing release of the hydrolytic enzymes into thecytoplasm which leads to muscle damage has been proposed (Kar and Pearson, 1979). They found the level of thiobarbituric acid-reactive products and the specific activities of catalase and glutathione reductase were significantly higher, while SOD activity was normal in DMD muscle. The increases in the lipid peroxidation-protective enzymes (catalase and glutathione reductase) may reflect a response to the increased lipid peroxidation in muscle.

Other workers have studied various proteinases and other hydrolases and their possible role in sarcolemmal disruption (muscle degeneration). Necrosis of muscle fibres in DMD was found to be due to an increased rate of proteolysis whilst protein synthesis was not impaired

(Kar and Pearson, 1976; 1978; 1980). They demonstrated increased activities of lysosomal cathepsins (most of them probably originating from the proliferating connective tissue, fat cells and macrophages), other lysosomal acid hydrolases, neutral proteinase, a Ca^{2+}-activated neutral proteinase, a chymotrypsin like esterase, alkaline proteinase (for which myoglobin was substrate) and dipeptidyl peptidase IV (probably microsomal) in muscular dystrophies, including DMD. They suggested that these phenomena were a nonspecific feature of degenerating muscle and usually parallel with the severity of muscle damage. Also they emphasized the important role of Ca^{2+}-activated proteinase in muscle breakdown, implying a membrane defect in Ca^{2+} transport. In their later work (1982), they found a thiol proteinase in muscle homogenates from muscular dystrophies which was significantly increased and proposed it also might be involved in the muscle wasting process.

To summarise, then, there have been many reports of increased catabolic enzyme activities (lipase, proteinase) in DMD muscle. Increased activities of the membrane lipid peroxidation protecting enzymes (catalase, glutathione reductase) may reflect the increased lipid peroxidation in the muscle membrane, which may also lead to membrane damage. The results may explain the process of muscle degeneration, but say little about the underlying primary defect.

Little agreement about the activities of membrane-bound enzymes is found in DMD RBC and muscle, but reports of increased lipase activity in both DMD RBC and muscle are noteworthy. Any change in membrane bound enzyme activity may be due to a common defect, for example, a change in their lipid domain in their immediate vicinity. If the membrane degeneration of DMD muscle is due to (or at least, in part) the increased activity of lipases, it is interesting that no gross membrane degeneration occurs in DMD RBC.

1.4.6 Findings in Tissues Other Than Muscle Biopsies and RBC

1.4.6.1 Lymphocyte Capping

Normally, membrane proteins (such as Ig-receptors) are uniformly dispersed across the lymphocyte surface and are anchored by microtubules. In response to certain stimuli, (e.g. drugs, temperature) the proteins will move from the dispersed state through various stages of aggregations (clusters) to an asymmetric distribution of larger aggregates (patches) and finally into a large single aggregate (cap) sitting on the cell surface. This process may be observed microscopically after labelling cell surface antigens (which are spanning proteins) with fluorescence antibodies. This capping technique permits one to observe the lateral mobility of integral membrane protein in the plane of the cell surface. Ca^{2+} and the membrane associated contractile elements, (the micro-filaments and the anchoring microtubules) may influence

the specific cell membrane protein to migrate to one pole of the cell, forming the cap. It may be assumed that a large number of factors can bring about surface Ig-receptor-antibody mobility changes, including abnormalities in membrane conformation and composition, and in the availability of energy in the form of ATP. Thus, measurement of membrane protein mobility appears to be a sensitive indicator of a multitude of possible intracellular and associated cell surface changes (Pickard, Grauemer, Verrill, Isaacs, Robinson, Nance, Myers and Goldsmith, 1978).

Pickard et al. (1978) incubated lymphocytes with fluorescein-labelled polyvalent anti-human immunoglobulin and found diminished cap formation in B and T lymphocytes from both patient and carriers. The differences are indistinguishable between patients and carriers. Similar results were obtained by Verrill and Pickard (1977); Nordal, Anderson and Dietrichson (1982) and Ho, Reitter, Stojakowits, Fiekn and Weisser (1980).

However no abnormality in lymphocyte capping was found by Fitzsimmons, McLachlan and Reever (1980); Stern, Kahan, and Dubowitz (1979) and Hauser, Weiner, Ault and Unanne (1979). Stern et al. (1979) explained the discrepancy in result in term of the difficulty in evaluation of lymphocyte capping. No further reports have been forth coming to solve this controversy. Therefore, at present, lymphocyte capping is not the reliable carrier detection technique as it was first proposed.

1.4.6.2 Muscle Tissue Culture in DMD

A later onset of growth of DMD muscle in tissue culture has been observed in some laboratories (Kakulas, 1968; Bateson, Hindle and Warren, 1972; Morgan, Cohen and Cohen, 1973). But O'Steen (1963) and Thompson (1977) found no abnormality in the growth characteristics of DMD muscle tissue culture. The size of the myotubules was demonstrated to be larger by Geiger and Garvin (1957) and Kakulas (1973) while O'Steen (1963) indicated shorter and wider myotubules in his DMD muscle tissue culture. Numbers of nuclei in the myotubules in the cultured DMD muscles were found to be increased (Geiger and Garvin, 1957) whereas another report (Morgan et al. 1973) showed a decrease. Both Morgan et al. (1973) and Vassilopoulos, Emery and Gordon (1977) showed larger nuclei in the DMD cultured muscle.

An increased cell stickness was demonstrated by Yasin (1979). She found an increased number of nuclear overlaps because the cells stick on top of one another to form cell clusters and proposed a number of molecules might be implicated in cell-cell interactions, cell-substractions interaction and cytoskeleton-membrane interactions. These surface-associated elements include collagen, fibronectin, glycosaminoglycans, lectins, desmin and gangliosides. But Ecob-Johnston and Brown (1981) found a normal membrane surface adherence in cultured DMD muscle while the DMD cells, like normal

culture did not form multilayered clusters. Also Mawatari, Miranda and Rowland (1976) and Ionasescu (1976; 1979) showed no morphological difference in their DMD cultured muscle cells when compared to control.

Few abnormalities of growth or cytology of DMD muscle in culture have been reported, therefore, and those which have are either unconfirmed observations from a single laboratory or are the subject of contrary reports by other workers.

1.4.6.3 Cultured Skin Fibroblasts in DMD

DMD cultured skin fibroblasts are reported to exhibit increased doubling time and a tendancy to be more voluminous cells (Liechti-gallati, Moser, Siegrist, Wiesmann and Herschkowitz, 1981) whereas Pena, Vust, Tucker, Hamenton and Wrogemann (1978) found normal growth characteristics and viability.

Other workers have studied membrane surface characteristics of the cultured DMD fibroblasts. Jones and Witkowski (1979) found no gross difference in cell surface morphology. In a later report (1981) they suggested there might be an alteration in the surface of dystrophic cells by studying the aggregation of the cultured fibroblasts in a Couette viscometer. They found the cells have a low intercellular adhesiveness and formed small numbers of very large aggregates which were absent in normal cells. Observed under the electron microscope the DMD cultured fibroblasts revealed the presence of abundant lamellar bodies, a morphologic abnormality

commonly associated with impaired lysosomal function (Gelman, Davis, Morris and Gruenstein, 1981). One might expect the abnormal surface characters of the DMD cultured fibroblasts to reflect some abnormality in their membrane proteins and/or lipids. Newman (1982) indicated a normally functioning cytoskeleton by staining with concanavalin A. Pena et al. (1978) demonstrated a normal distribution of subcellular fractions and normal major protein components. A recent study (Burghes, Dunn, Statham and Dubowitz, 1982) of DMD cultured skin fibroblasts protein by SDS-gel electrophoresis showed the regions of 73,000-68,000 and 48,000 MW were decreased while those of 175,000 and 53,000 MW were significantly elevated.

No abnormality was found in the phospholipid profiles and fatty acid composition of DMD cultured fibroblasts of either young or old cultures (Kohlschütter, Weismann, Herschkowitz and Ferber, 1976). But Rounds, Jepson, McAllister and Howland (1980) found that the concanavalin-A induced increase in ^{32}P incorporation into phosphatidic acid (PA) and decreased label in PI was significantly greater in DMD skin fibroblasts. They suggested there was a diminished rate of PI resynthesis from PA. But the activities of the two transferases involved were found to be normal, so theyproposed that there might be a deficiency in acylation of PA to give PI.

Willers (1982) showed a normal 5'-nucleotidase activity in DMD cultured skin fibroblasts. But

Liechtigallati et al. (1981) indicated an elevated 5'-nucleotidase activity, and together with an increased doubling time in DMD cultured fibroblasts, they suggested the DMD cells behaved similarly to prematurely aging cells. Adenylate cyclase activity was not different from control in DMD cultured fibroblasts (Cerric, 1982). An alteration in the properties of the lysosomal membrane in DMD cultured fibroblasts was proposed by Gelman, Davis, Morris and Gruenstein (1981). Treatment of lysosomes with the nonionic detergent Triton X-100 causes an activation of a membrane-bound lysosomal enzyme dipeptidyl amino-peptidase-1. This activation was markedly diminished in cultured DMD fibroblasts. Ca^{2+} transport in cultured DMD fibroblasts was studied by Statham and Dubowitz (1979). They found no difference in calcium exchange studied by ^{45}Ca and a normal increase of Ca^{2+} exchange induced by calcium ionophore A 23187.

Although relatively little work has been done on cultured skin fibroblasts, so that most of the reported abnormalities have not been tested in other laboratories yet, a membrane defect appears possible, especially in view of the observations on adhesiveness. It would seem timely to investigate all surface glycoproteins and glycolipids in more detail.

1.5 Aim of This Work

Much evidence, although some controversial suggests that the primary defect of DMD is a systemic membrane abnormality which is manifest in other tissues as well as muscle. A defect in lipid organization or metabolism seems a possible and attractive unifying explanation of the observed abnormal membrane and membrane-associated properties. As several reports have already shown that membrane lipid composition is normal in the disease, the aim of this investigation was to examine the possibility of a perturbed membrane lipid asymmetry in the bilayer in RBC from patients with DMD, using phospholipase A_2 from bee venom to hydrolyze the outer layer lipids in intact RBC.

2. MATERIALS AND METHODS

2.1 General

Glassware

Prior to use, all glassware (including Pasteur pipettes, TLC tanks) were soaked overnight in 5% Decon 90 (Decon Laboratories, Brighton, England). Decon 90 is a phosphate-free detergent which is important since most laboratory detergents contain high levels of phosphate and can lead to contamination of the phosphorus assay.

Solvents

All organic solvents (except glacial acetic acid) were redistilled and contained the antioxidant 2,6-ditert-butyl-p-cresol (butylated hydroxytoluene, BHT, Sigma) 50mg/L. BHT does not interfere with the chromatography or subsequent analysis (Christie, 1976).

Other Chemicals

Glacial acid A.R. and disodium EDTA were from May and Baker. 72% perchloric acid (Anala R), ammonium molybdate and Tris were from BDH. Authentic standards PC, LPC, PE, LPE, PS, LPS and SM were from Sigma. Lyophilised bee venom (Apis mellifera) phospholipase A_2 (EC 3.1.1.4) was from Sigma and was made up in 50% glycerol to 200 units/ml and stored at -20°C (solution does not freeze, and retains activity for 1-2 years). (Roelofsen and Zwaal, 1977). The enzyme was stated (by Sigma) to have a minute amount

of proteinase activity, and gave a single band on SDS-PAGE but was not purified prior to use.

TLC Plates

20 x 20 cm precoated with 0.25 mm thick Silica gel 60 were from Merck.

The Patients

All the patients are definite DMD according to their family history, CPK test, muscle histology and electrocardiogram. The patients' ages ranged from 14-22 whilst those of controls (2F, 8M) were 20-42.

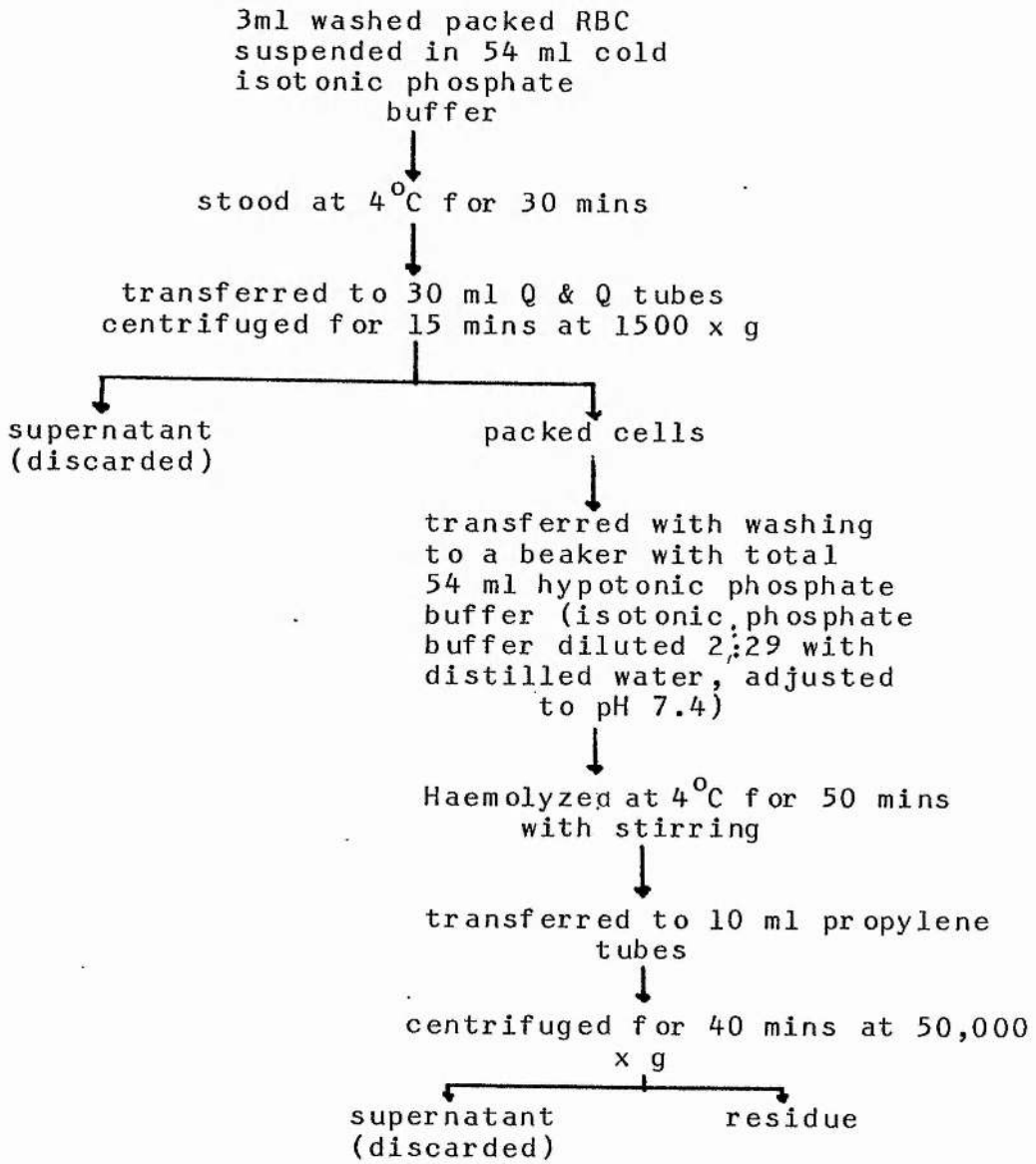
2.2 Isolation of Washed Whole Erythrocytes

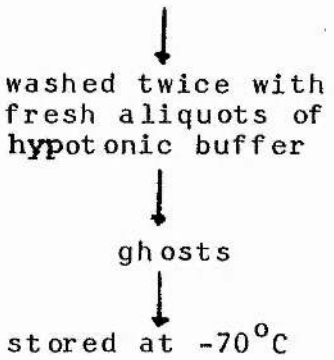
10 ml of blood was drawn from a precubital vein into a 10 ml heparin-precoated tube. The contents were mixed by gentle shaking. The blood was centrifuged at 1500 x g for 10 mins and the plasma aspirated off. The packed RBC were transferred to 30 ml Q and Q tubes and resuspended in an equal volume of buffered isotonic saline (0.87% NaCl, 0.25 mM $CaCl_2$, 0.25 mM $MgCl_2$, adjusted to pH 7.4 with 0.05 M Tris). The cells were centrifuged at 1500 x g for 15 mins and the buffy coat (white cells and platelet aggregates) removed along with the top 2 mm of red cells (consisting of damaged erythrocytes). The packed red cells were washed twice more with fresh aliquots of the same buffer. 10 ml of blood produced about 3.5 ml of washed packed RBCs.

2.3 Isolation of Ghosts

The method was modified from Dodge, Mitchell and Hanahan (1963) with extending the haemolytic time from 10 mins to 50 mins. Red cells were washed twice with isotonic phosphate buffer (pH 7.4, 0.103 M Na_2HPO_4, 0.155 M NaH_2PO_4) in order to minimize the amount of Ca^{2+} which will promote the endogenous breakdown of phospholipids. The procedure is shown in the following figure.

Ghost Preparation




washed twice with
fresh aliquots of
hypotonic buffer
ghosts
stored at -70°C

2.4 Treatment of Intact RBC with Phospholipase A_2

The method was modified from Roelofsen and Zwaal (1977). Cells were treated with the enzyme within 2 hrs of sampling to minimize haemolysis and other possible changes in membrane organization known to occur with storage. In order to optimize the condition to obtain maximal degradation of the phospholipids at the outer layer of RBC accompanied by minimal haemolysis several variations in procedure were investigated.

1. Amount of enzyme added.

5U, 10U, 15U and 20 U of the enzyme were added per 0.2 ml packed cell.

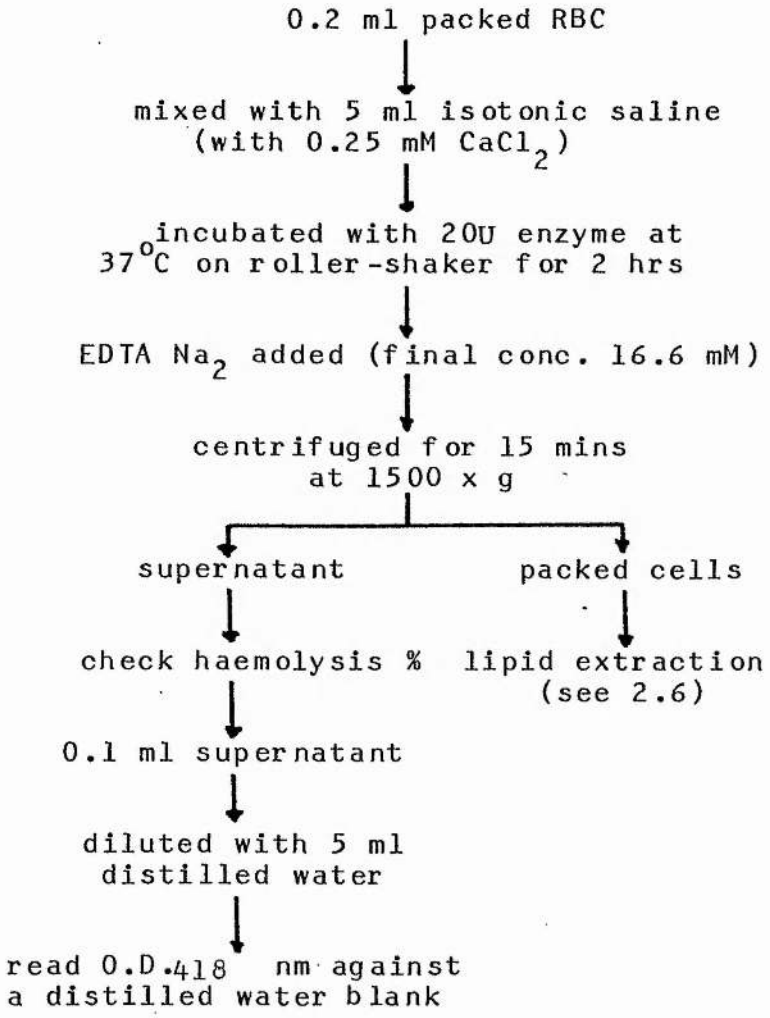
2. Length of incubation time

The cells were incubated with enzyme for 1 hr, 1.25 hrs, 1.5 hrs, 2 hrs and 2.5 hrs at 37°C on a roller-shaker.

3. Concentration of Ca^{2+} and EDTA

Both 10 mM $CaCl_2$ and 0.25 mM $CaCl_2$ were used in the incubation and washing buffers. EDTA Na_2 (in the isotonic saline) was added to stop the enzyme-incubation at final concentrations of 8.33 mM and 16.6 mM.

Final Procedure



A control with no enzyme added was incubated for the same time to determine the extent of any endogeneous phospholipase activity. 100% haemolysis was determined by suspending 0.2 ml packed cells in 5 ml distilled water. The $O.D._{418}$ nm of the supernatant from this second control was defined as 100% haemolysis. Progress of haemolysis with time was also examined by incubating 0.2 ml packed RBC in 5 ml saline with 20 U enzyme. 1 ml extracts were taken every 15 mins to determine haemolysis.

2.5 Treatment of Ghosts with Phospholipase A_2

The method of Roelofsen and Zwaal (1977) was modified. The ghosts (derived from 0.2 ml of packed RBC) were suspended in 5 ml of Tris buffer (0.1 M Tris, 0.25 mM $CaCl_2$, 0.25 mM $MgCl_2$, adjusted to pH 7.4) and incubated with 20 U of phospholipase A_2 at 37°C for 2 hrs on a roller-shaker. At the end of incubation 100 mM EDTA Na_2 (final concentration 16.6 mM) was added to stop the enzymatic activity. The suspension was centrifuged for 40 mins at 50,000 x g. The supernatant was removed and the pellet subjected to lipid extraction (2.6).

A control (without enzyme) was treated identically. As SM is not the substrate of the enzyme, it is used as an internal standard. SM% and degradation % are calculated by the following formula:

$$SM\% = (\text{amounts of PC or PE})/\text{amount of SM} \times 100$$

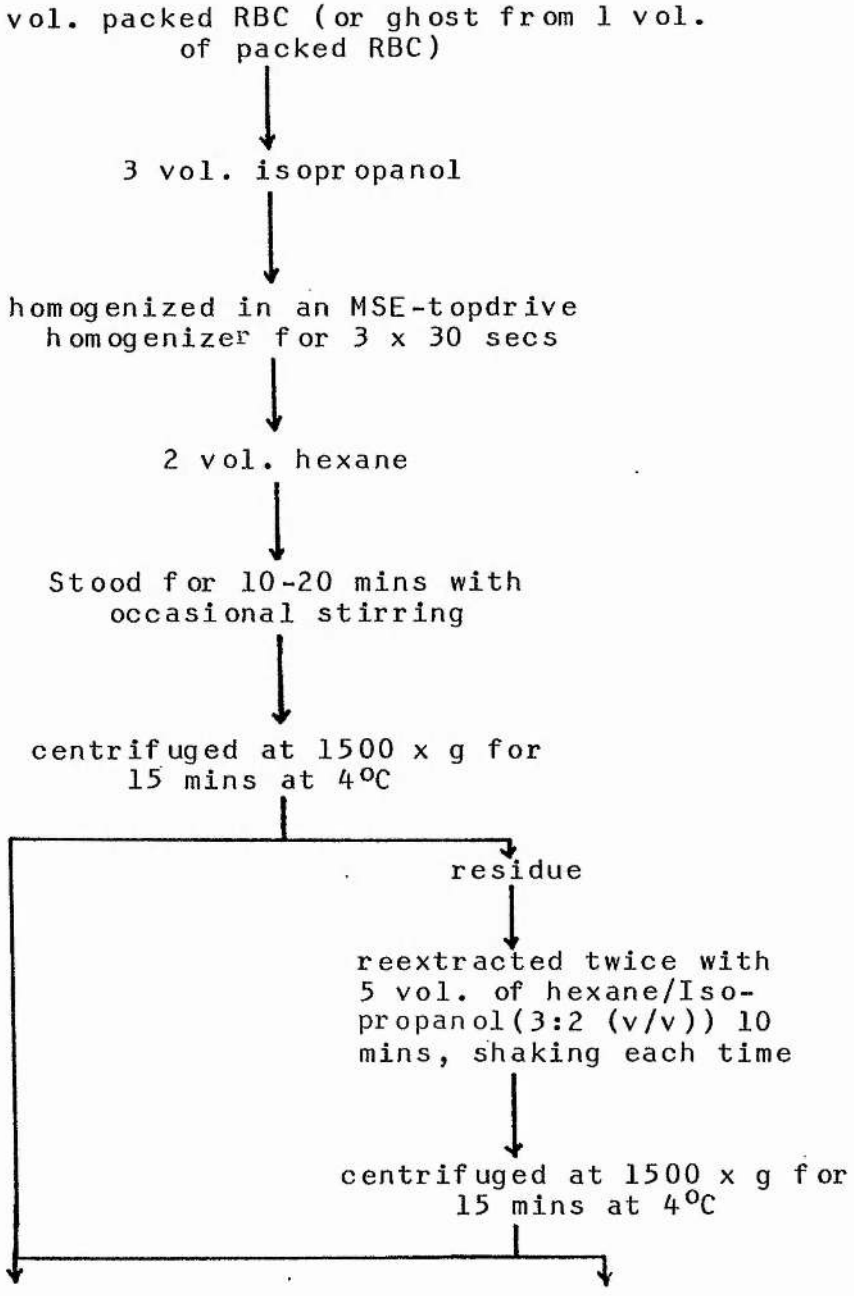
$$\text{Degradation\%} = \frac{\text{SM\% of PC (or PE) of control sample} - \text{SM\% of PC (or PE) of enzyme-treated sample}}{\text{SM\% of PC (or PE) of control sample}} \times 100$$

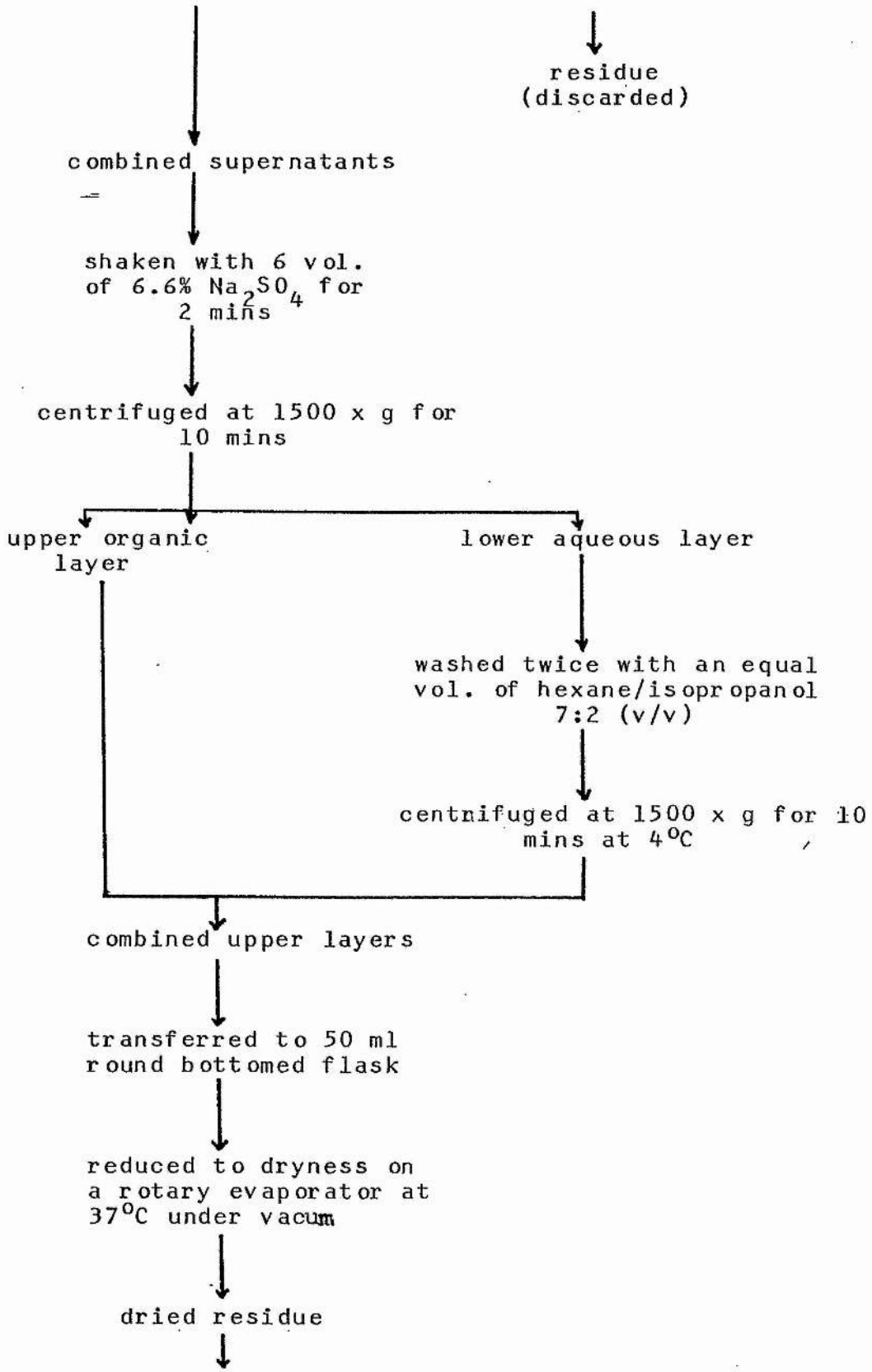
2.6 Lipid Extraction

Five methods of lipid extraction were tested for their efficiency.

1. Hexane:Isopropanol 3:2 (v/v)

(Hara and Radin, 1978)




residue
(discarded)
combined supernatants
shaken with 6 vol. of 6.6% Na_2SO_4 for 2 mins
centrifuged at 1500 x g for 10 mins
upper organic layer
lower aqueous layer
washed twice with an equal vol. of hexane/isopropanol 7:2 (v/v)
centnifuged at 1500 x g for 10 mins at 4°C
combined upper layers
transferred to 50 ml round bottomed flask
reduced to dryness on a rotary evaporator at 37°C under vacum
dried residue

↓

redissolved in
chloroform/methanol 1:1 (v/v)

↓

transferred quantitatively
to a small vial

↓

reduced to dryness on a heating
block under a stream of N_2

↓

redissolved in chloroform/
methanol 1:1 (v/v) to give a
concentration of lipid equivalent
to 1 ml packed cell in 0.1 ml

↓

stored at -20°C for a maximum
of 24 hrs prior to chromato-
graphy

2. Chloroform/methanol 1:1 (v/v)

(Zwaal and Roelofsen, 1977)

0.25 ml packed cells was added to 5 ml of methanol and stirred for 5 mins. Then 5 ml of chloroform was added and stirred for another 5 minutes. The suspension was centrifuged at 1500 x g and the extraction repeated once more. The combined lipid extracts were concentrated as described above.

3. Chloroform/methanol 1:1 (v/v) and 2:1 (v/v)

(Broekhuyse, 1969)

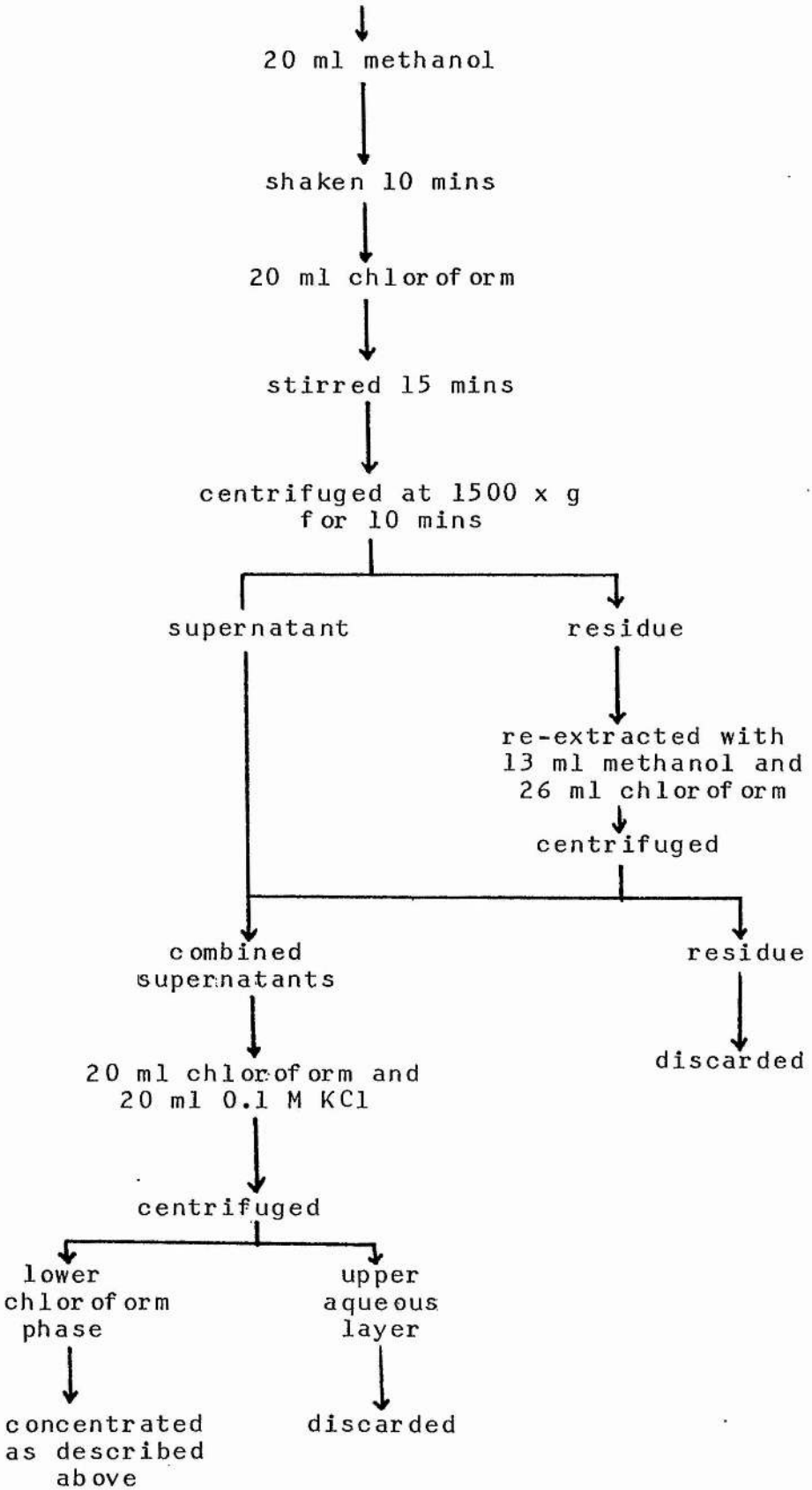
2.5 ml packed cells (or ghosts
from 2.5 ml packed cell)

↓

2.5 ml distilled water

↓

stood for 10 mins to
complete haemolysis


20 ml methanol
shaken 10 mins
20 ml chloroform
stirred 15 mins
centrifuged at 1500 x g for 10 mins
supernatant
residue
re-extracted with 13 ml methanol and 26 ml chloroform
centrifuged
combined supernatants
residue
discarded
20 ml chloroform and 20 ml 0.1 M KCl
centrifuged
lower chloroform phase
upper aqueous layer
concentrated as described above
discarded

4. Method 3 but haemolysis omitted.

*5. Method 4 but KCl washing omitted.

* Method 5 was finally adopted as giving the best recovery of all phospholipids.

2.7 2-D TLC of Phospholipids

TLC plates (precoated with silica gel 60H, 20 x 20 cm, 0.25 mm thick) were used. Prior to applying sample, the plate was activated in a 112°C oven for 1 hr. After the plate had cooled, the samples were applied with a microsyringe (Terumo graduated 10 µl, or Behring 20 µl dispensor) on to a small spot of rhodamine solution (0.00 12% in chloroform/methanol 1:1 (v/v)) in the bottom right hand corner of the plate, 1" from edges of silica. This spot served to pinpoint the origin for accurate loading of the samples. During development of the plate rhodamine also helped to locate the solvent front as it will run faster than all the lipids in both dimensions.

The plates were developed in two-dimensions in three solvent systems in an attempt to optimize resolution of the polar lipids.

All solvent ratios are v/v:-

1. Adapted from Roelofsen and Zwaal (1979) by Hunter (personal communication)

   First dimension:

   chloroform/methanol/ammonia (25% w/v)/water,
   80/44/5.5/5.5

   Second dimension:

   chloroform/methanol/glacial acetic acid/water
   100/54/12/2

2. Brophy (personal communication)

   First dimension:

   chloroform/methanol/ammonia (35% w/v)/H_2O
   290/174/12.7/25

Second dimension:

chloroform/methanol/acetone/glacial acetic acid/H_2O

400/130/150/120/80

*3. Adapted from 1.

First dimension: 1.

Second dimension:

chloroform/methanol/glacial acetic acid

50/27/12

* This solvent pair was finally adopted for the majority of the work.

Solvent mixtures were prepared daily to avoid possible chemical interactions between components and losses by evaporation. Tanks were washed with solvent and the sides of the tank lined with a 20 cm high strip of Whatman 3 MM paper, soaked with and dipping into the solvent to ensure a saturated atmosphere. The tanks were sealed by a strip of 2 inch wide parcel tape around the rim and were allowed to saturate for at least 1 hr at $4^{o}C$ before the TLC plates were inserted.

Duplicate TLC plates were developed at the same time. The solvent front was allowed to run within 1 cm (approximately) of the top of the plates. Development times (approximately) for system 3 were 2 hrs (1st dimension) and 3 hrs (2nd dimension).

Between runs in the different systems, plates were dried under a warm hair-dryer for about 15 mins. This step is critical for good separation. It is important that almost all traces of the first dimension solvents are removed.

2.8 Detection of Lipid Components

After developing the second dimension, plates were dried under a warm hair-dryer for about 20 mins. Three kinds of detection agents were tried:

1. Charring agent

The dried plates were sprayed with charring reagent (1.2 g $K_2Cr_2O_7$ in 200 ml 55% H_2SO_4 (Kates, 1978) and heated for 20 mins at 180^0C in a forced-draught oven to char the organic matter. The phospholipids should appear as black spots on a white background.

2. Rhodamine 6G

The dried plate was sprayed with aqueous Rhodamine solution (0.0012%) and examined wet under ultraviolet light (366 nm). Acidic phospholipids e.g. PS and LPS will give a purple colour, PC, PE and SM will appear as yellow spots while other neutral lipids appear pink.

3. Iodine Vapor

The dried plates were inserted into an iodine containing tank. The tank was gently warmed with a hair-dryer to hasten subblimation of the I_2. The phospholipids appear as brown-yellowish spots on a pale-yellow background. For reasons given later this method was adopted for routine detection.

2.9 Quantitative Analysis of Phospholipids (Rouser, 1969)

2.9.1 Standard Phosphorus Curve

Na_2HPO_4 solution (Stock, 5 µg phosphorus/ml) was used to prepare the standard curve. Appropriate volumes of solution were added to tubes containing 0.9 ml 72% perchloric acid and the total volume was made up to 2.9 ml with distilled water. To each, 5 ml of distilled water was added, followed by 1 ml 2.5% ammonium molybdate and 1 ml 1% ascorbic acid (freshly prepared). The test tubes, with marbles over the mouth to prevent evaporation, were heated in a boiling water bath for 5 minutes. A reagent bank was heated at the same time. The $O.D._{820}$ was read after the tubes had cooled to room temperature. The calibration series were done in duplicate. A plot of $O.D._{820}$ against µg of phosphorus was linear up to 10 µg phosphorus.

2.9.2 Digestion of Phospholipids

The optimum time of digestion of authentic phospholipids (in the presence of I_2 and silica) to ensure complete release of organic phosphorus was determined. Authentic standards of PS containing identical amounts (about 5 µg) of phosphorus were spotted onto a TLC plate. The spots were detected by iodine vapor (without running chromatography), scraped onto a piece of clean filter paper and transferred to a 30 ml Kjeldahl tube containing 0.9 ml 72% perchloric acid. The mixture was digested

under gentle reflux in an electrically-heated Kjeldahl rack within a fume cupboard. Vigorous boiling was avoided to prevent loss of perchloric acid and boiling off of any phosphoric acid formed. Digestion was continued for 10 mins, 20 mins, 30 mins, 40 mins and 50 mins. At these times tubes were removed, cooled in air for 15 mins and the walls of the tubes rinsed down with 5 ml distilled H_2O.

1 ml 2.5% ammonium molybdate, 1 ml 10% ascorbic acid and 2 ml distilled water were added as described before and the contents transferred to 10 ml centrifuge tubes. After heating in a boiling water bath for 5 mins, the tubes were centrifuged at 1500 x g for 10 minutes to precipitate the silica particles. The $O.D._{820}$ of the supernatant was determined after cooling. A curve of the recovery of PS against digestion time was plotted (graph showed in result section) and showed that a digestion time of 30 mins gave the highest recovery of PS.

2.9.3 Phosphorus Determination of Individual Phospholipid Classes

After the phospholipids spots were located by the detection reagent (in duplicate), each individual spot was scraped onto a piece of clean filter paper and digestion performed as described in 2.9.2. A blank spot of the same size as the individual phospholipid spots was also scraped off to determine the background phosphorus content of the plates. A standard was always included in an estimation to compensate for daily variations in reagents, conditions, etc.

2.9.4 Recovery of Phosphorus from TLC Plates

Aliquots of authentic SM standard containing about 5 µg of phosphorus were placed inside the 30 ml Kjeldahl tube and evaporated to dryness. The contents were digested for 30 mins with 72% perchloric acid and phosphorus determined as described above. This served as a control to indicate the total phosphorus in the sample. The same amounts of SM were spotted onto a TLC plate and separated by 2-dimensional chromatography, stained and scraped off. Digestion and phosphorus determination were performed as described before. The phosphorus recovered after TLC was expressed as a percentage of the total SM loaded.

2.10 Incubation of Normal RBC with DMD Plasma

Lloyd and Emery (1981) incubated normal erythrocyte ghosts in Duchenne plasma, determined the (Na^+, K^+)ATPase activity and found an increased enzyme activity on subsequent exposure to ouabain i.e. normal cells could be made "Duchenne-like" by incubate with Duchenne plasma. They suggested there might be a circulating plasma factor in DMD responsible for the abnormal response to ouabain. In order to investigate the possible existence of a 'circulating plasma factor' which may influence the lipid bilayer organization of the normal RBC incubation of normal RBC with DMD plasma was done.

1.5 ml of normal packed cells were mixed with 0.75 ml of plasma from patients with DMD and then incubated for 2 hrs at 37°C. At the end of incubation, 30 ml of isotonic saline were added. The supernatant was removed by suction after centrifugation for 15 mins at 1500 x g. Phospholipase A_2 digestion of whole cells (plus an appropriate control) and analysis of phospholipids was then carried out as before.

3. RESULTS AND DISCUSSION

3.1 Lipid Extraction Methods

Five methods of lipid extraction were compared for their efficiency in extracting phospholipid classes from whole erythrocytes. The results are shown in Table 8. A typical thin-layer chromatogram of total red cell lipid extracted by method II is shown in Fig. 1.

Membrane lipid extraction is usually accomplished by the use of a mixture of a relatively non-polar solvent, such as chloroform which will disrupt hydrophobic bonds and a more polar species such as methanol which will also disrupt other lipid - protein interaction, such as hydrogen bonds as well as acting as a protein denaturant. Chloroform-methanol mixtures are usually the solvents of choice, often in the proportions recommended by Folch, Lees and Stanley (1957), which comprised a chloroform/methanol (2:1 v/v) and a wash (water or salt solution such as KCl). Hara and Radin (1978) recommended a new solvent system: hexane/isopropanol, followed by washing the extract with aqueous sodium sulfate which can remove nonlipids, and a back wash of the aqueous layer with H/IP 7:2 if complete recovery of the lipids is needed. Although they claimed this extraction method was only inefficient for the extraction of gangliosides, the results of this work showed a very low recovery of PS and of total phospholipid compared with the literature. One reason may be the sticky mass formed after the addition of isopropanol (in

Table 8 Extraction Methods (see 1.2.)

Phospholipid	I (n=3)	II (n=3)	III (N=3)	IV (n=3)	V (n=3)
PC	32.4±0.9	28.6±0.6	30.2±0.1	30.4±0.7	29.6±0.2
SM	23.6±1.9	27.1±0.2	29.2±0.4	24.4±2.2	28.0±0.8
PS	3.4±0.6	9.2±1.9	8.0±0.5	11.0±1.3	11.0±0.7
PE	39.7±2.4	29.7±1.3	28.8±0.3	28.0±0.9	30.3±0.4
LPC	Tr.[a]	0.8±0.5	1.1±0.5	0.8±0.4	Tr.[a]
DPI	N.D.[b]	N.D.[b]	N.D.[b]	1.6±0.9	Tr.[a]
PI	Tr.[a]	0.7±0.2	0.8±0.1	0.7±0.2	0.4±0.4
PA	0.8±0.2	1.3±0.8	1.3±0.1	1.7±0.4	0.5±0.5
Origin	N.D.[b]	2.5±1.0	0.6±0.1	1.4±0.4	Tr.[a]
yield (µ moles lipid P/ml packed cell)	1.84±0.15	3.11±0.26	2.89±0.09	3.40±0.06	3.55±0.44
Recovery of P after TLC (%)	89.4±4.0	90.5±5.6	96.1±2.5	90.4±1.8	98.9±5.2

Remarks:

Tr.[a] Detectable by I_2 vapour but below detection limit of colorimetric P assay, less than 0.3% total lipid P.

N.D.[b] Not detectable on TLC.

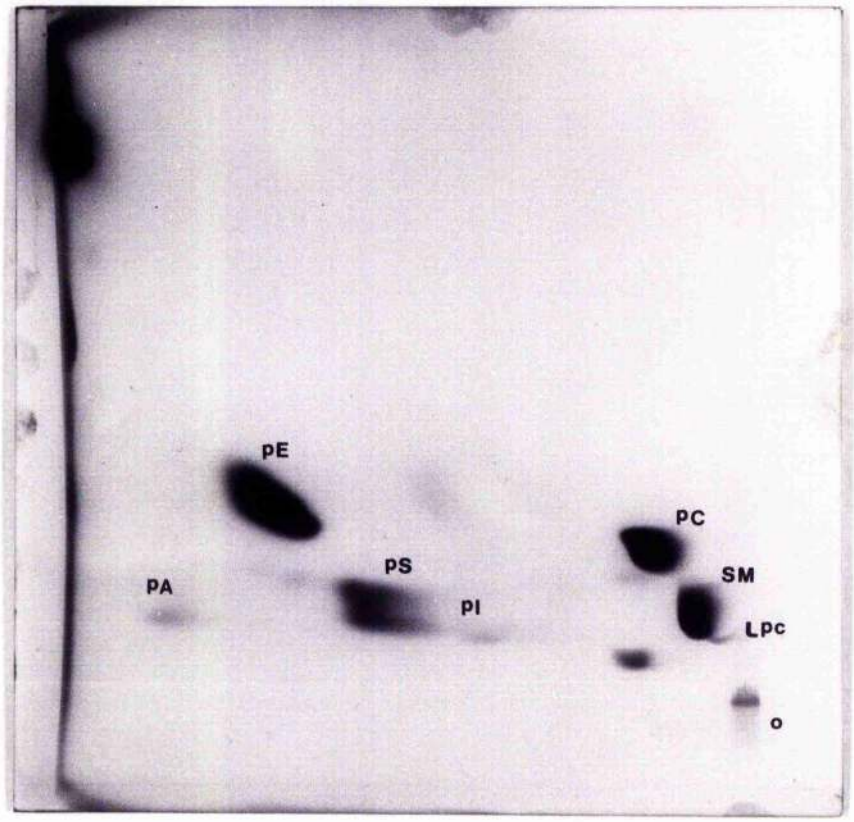


Fig. 1 Red Cell Lipids Extracted by Method II (see 2.6.). Loading: Lipid from 0.3 ml Packed Cells.

our hands) which limited the access of the solvent to the membrane material, and the aqueous washing step. The aqueous washing procedure will remove most of the gangliosides and ceramide polyhexosides in the original extract. Not only are these carbohydrate - containing lipids lost in the aqueous wash, but the acidic phospholipids, such as PS, PA and PI are carried into the aqueous phase (Nelson, 1972). The disadvantage of a washing procedure in this respect are shown by comparing the results of method III and method IV. Higher levels of PS, DPI and PA were found using method IV than method III, in which the only difference was the inclusion (method III) and absence (method IV) of the KCl wash.

Chloroform/methanol (1:1 v/v) and reextraction with the same solvent system was used in method II which gave a relatively low PS recovery and a large amount of heme (large heme spot on TLC). Nelson (1972) recommended a change in the solvent system for re-extraction as the recovered proportions of lipids are the same if using the same solvent system for re-extraction. In method V, re-extraction using a different solvent system (C/M 2:1) gave a greater recovery of PS and total phospholipid and a relatively small amount of heme. Heme is known to catalyze autoxidation of unsaturated lipids (van Deenen and De Gier, 1974) which will lead to a loss of polyunsaturated lipids, such as PS and PE and a relative increase of the more saturated fractions, such as SM and PC (Dodge and Phillips, 1966).

Comparing the results for IV and V it is apparent that neither omitting the initial haemolysis step nor increasing the solvent: cell ratio improves the extraction efficiency. It is not necessary to grind or disrupt the cells if they are added dropwise to methanol with stirring (Nelson, 1972), which was done in this work. Although heavy losses of lysophospholipids have been reported to occur when chloroform-methanol mixtures are used, and butanol extraction is suggested to overcome this (Bjerve, Daae and Bremer, 1974), the recoveries of lyso-derivatives after phospholipase A_2 treatment of intact cells and ghosts in this work were good using method V (results shown in later section).

It is concluded from this study that, where maximum recovery of total phospholipid, and especially of acidic classes, is required from erythrocytes, the method of Broekhuyse is recommended, but with the omission of the KCl wash. This will obviously lead to the presence of non-lipid contaminants in the extract but these do not interfere with the TLC and the colorimetric estimation after chromatography. Phosphorus-containing non-lipid contaminants, however, may account for the rather low ($\sim$ 85%) recovery of lipid P after TLC found sometimes in this work.

Method V for lipid extraction was used for all subsequent experiments. A comparision of the red cell lipid composition obtained using method V and two acceptedly reliable literature values are shown in Table 9.

Table 9 Red Cell Lipid Composition in this Work and Literature (expressed as % phosphorus w/w)

Phospholipid	(n=3) This work	Broekhuyse (1969)	Dodge and Phillips (1967)
PC	29.6±0.2	28.3±2.1	29.2±1.5
SM	28.0±0.8	25.8±1.7	25.4±1.4
PS	11.0±0.7	12.7±1.3	14.8±1.7
PE	30.3±0.4	26.7±1.0	27.5±1.5
LPC	Tr.[a]	1.4±0.8	1.0±0.8
DPI	Tr.[a]	1.0±0.8	-
PI	0.4±0.4	1.9±0.6	0.6±0.5
PA	0.5±0.5	1.0±0.2	1.1±0.5
Origin	Tr.[a]	-	0.4±0.6
Yield (μ moles lipid P/ml packed cell)	3.55±0.44	3.95±0.26	3.9±0.23
Recovery of P after TLC (%)	98.9±5.2	96.4±3.9	94.5±4.0

Remark:

Tr.[a] Detectable by I_2 vapour but below detection limit of colorimetric P assay, less than 0.3% total lipid P.

3.2 Solvent Systems for TLC

Thin-layer chromatography was achieved at 4°C using precoated silica gel 60 (Merck) plates. Low temperature can improve resolution by minimising diffusion of spots and help prevent autoxidation of polyunsaturated species, such as PS and PE, which can lead to losses (Dodge and Phillips, 1966).

Three solvent systems were used to separate standard phospholipids. Typical chromatograms are shown in Figs. 2, 3 and 4.

Fig. 2 shows lipids separated by solvent system I from Zwaal and Roelofsen (1977) modified by Hunter. There is good resolution of PC, SM, LPC, LPS, PE and PA, but PS gives a large diffuse spot and always overlaps with LPE and PI. Fig. 3 shows the chromatogram obtained with solvent system II from Brophy (personal communication). This gives good separation of PE, PC, PS, LPC, SM, PA and PI. But the disadvantage of this solvent system is overlap of LPS and LPE with PC, SM, PS and PI. Fig. 4 shows another modification from Zwaal and Roelofsen (1977) (solvent system III); PC, SM, LPC, PE and PA are in similar positions to Fig. 1, but PS become more condensed and moved faster in the 2nd dimension. This gives a better separation from LPE. LPS overlaps with a glycolipid but this does not interfere with following phosphorus determinations. As it was necessary to resolve all the major phospholipid classes and their lyso-derivatives (following phospholipase A_2 treatment), solvent system III was used for all subsequent experiments.

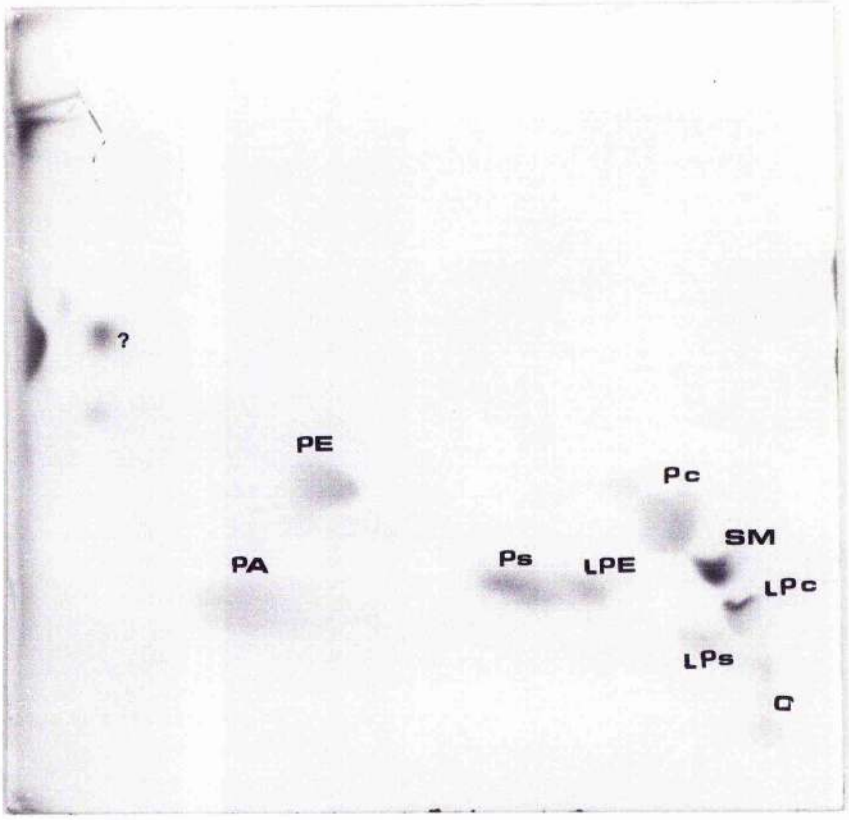


Fig. 2. Phospholipid Standards Separated by Solvent System I (Zwaal and Roelofsen (1977) Modified by Hunter) (see 2.7.). Loadings: 5 µg of Phosphorus in each Phospholipid.

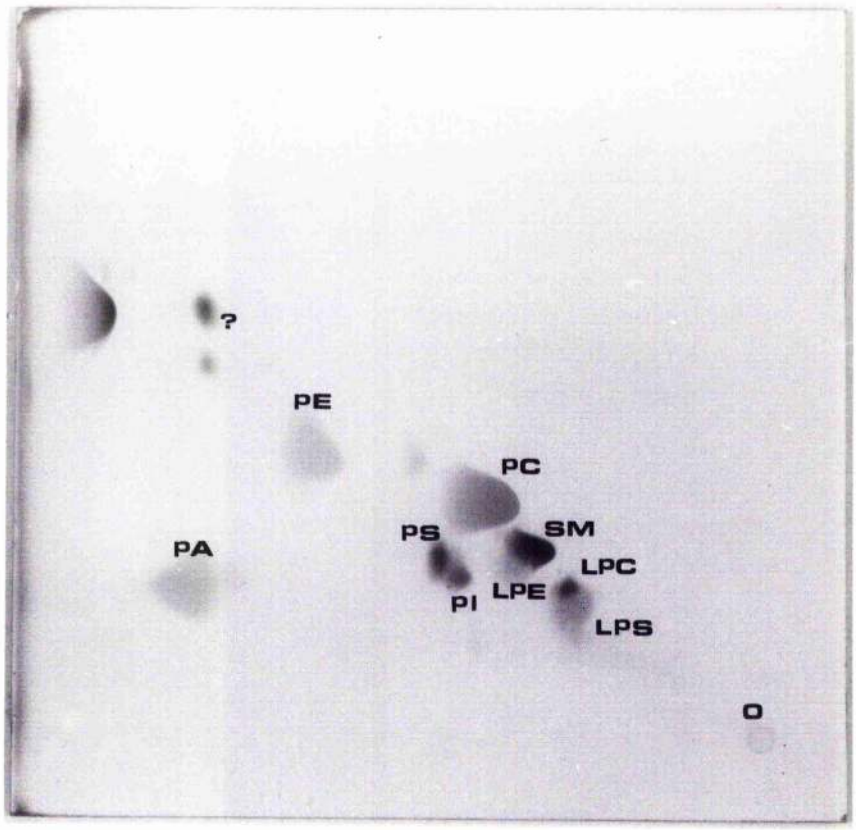


Fig. 3. Phospholipid Standards Separated by Solvent System II (Brophy, Personal Communication) (see 2.7.). Loadings: 5 µg of Phosphorus in Each Phospholipid.

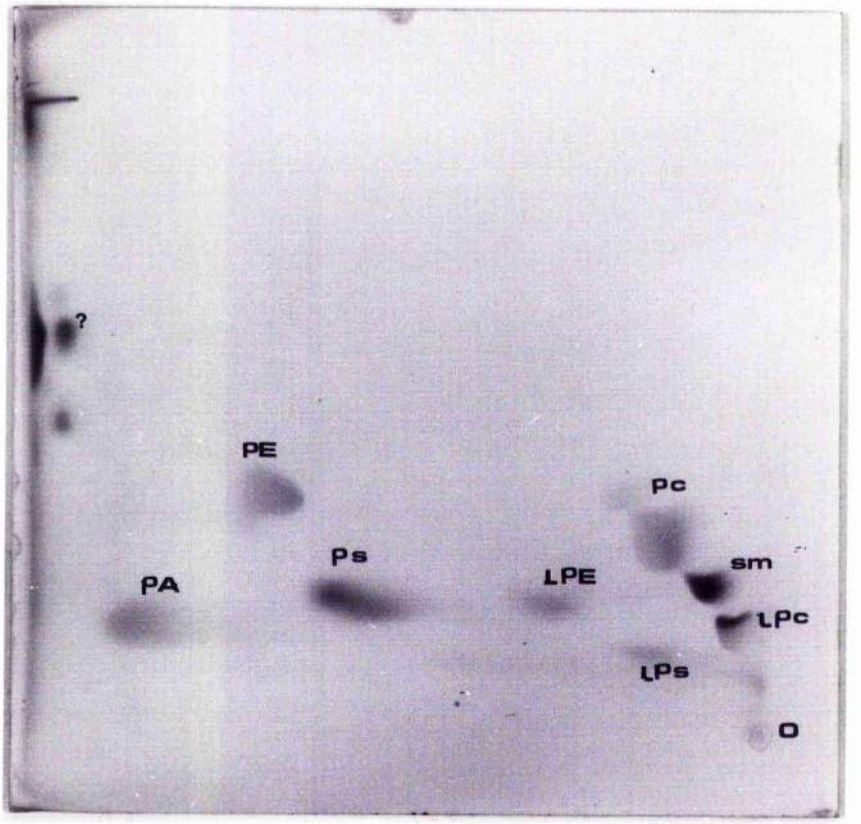


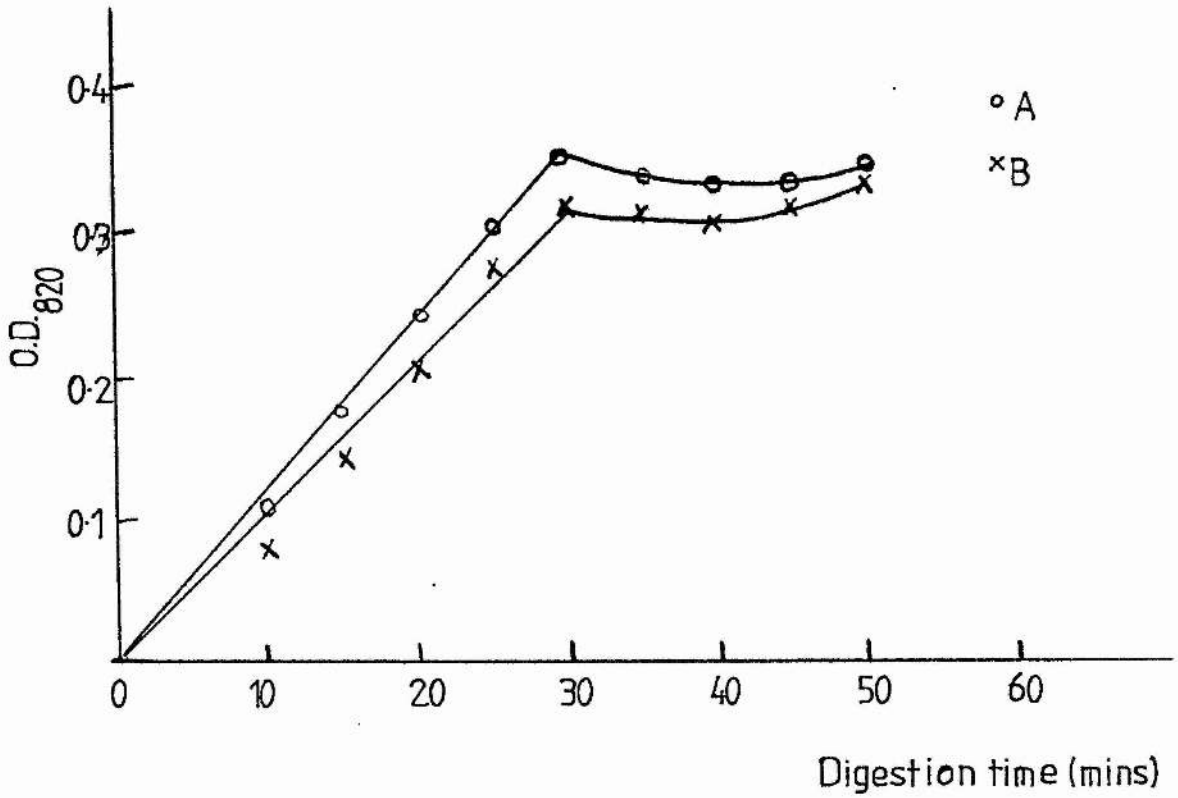
Fig. 4. Phospholipid Standards Separated by Solvent System III (Another Modification from that of Zwaal and Roelofsen (1977)) (see 2.7.). Loadings: 5 µg of Phosphorus in each Phospholipid.

3.3 Detection Methods

Three kinds of detection methods, i.e. rhodamine, charring and iodine, have been used to detect the lipid spots on TLC. Neither rhodamine nor charring gave consistent results. Rhodamine is not as sensitive a method as the other two and may lead to some loss of minor lipids and of small amounts of lipid at the periphery of spots. Charring is very sensitive and reacts with all lipids, but some losses, especially of PS, were found. The plate is heated in a 190^{o}C oven after spraying with charring agent, and oxidation products of the organic phosphorus, e.g. phosphoric acid may be volatilised which lead to some losses. The extent of reaction of different lipids with sulfuric acid may be different which will give inconsistent results. Iodine is a sensitive method which again detects all lipids. The procedure is simpler than the other two detection methods and no losses were found. Therefore, this method was used in all subsequent experiments.

3.4 Time Course Test of Release of Lipid P by Digestion with Perchloric Acid

Standard PS was used in this experiment to determine optimal digestion time. The amount of P recovered was linear with time up to 30 mins. and 30 mins. was found to be the optimal time for both PS samples digested directly and those recovered from TLC plates (in the presence of adsorbant but without actual chromatography). Interestingly, for digestion times longer than 50 mins., the subsequent colorimetric P method gave a dark-blue colour independent of the phosphorus content. This may be due to change in acidity, since prolonged digestion will result in loss of some perchloric acid. With insufficient perchloric acid present, molybdate seems to be directly reduced to a blue colour independent of the phosphorus content. This was testing by mixing ammonia molybdate, ascorbic acid and an appropriate amount of water, boiling for 5 mins. after which the solution turned dark blue even though no phosphorus was present. This time course curve is shown in Fig. 5.



A. Pure PS

B. PS and adsorbant from TLC plate

Fig. 5. Time Course Experiment of PS digestion.

3.5 Standard Phosphorus Curve and Calculation of Results

Two separate assays were performed. The results of two standard P estimations are shown in Fig. 6. The slope was plotted after determination by linear regression using the mean data from both assays. The slope of the graph was found to be constant from day to day.

$O.D._{820}$ converts to µg phosphorus as follows:

$$\mu g\ P = O.D._{820} \times \frac{1}{0.124666}$$

For conversion to µg of phospholipids, the multiplication factor is 25 for PC, PS, PE and SM and 16 for lyso-derivatives, since the proportion of phosphorus is 4% and 6% w/w respectively.

The increase in $O.D._{820}$ was found to be linear up to 10 µg of phosphorus. However, the maximum amount of phosphorus per spot was always adjusted to about 5 µg to ensure it was within the linear portion of the curve and on the most efficient range of the spectrophotometer. A phosphorus standard was run together with all the samples in each experiment to ensure the accuracy of phosphorus determination and minimise the daily variability of the reagents (especially ascorbic acid which was prepared freshly at each experiment).

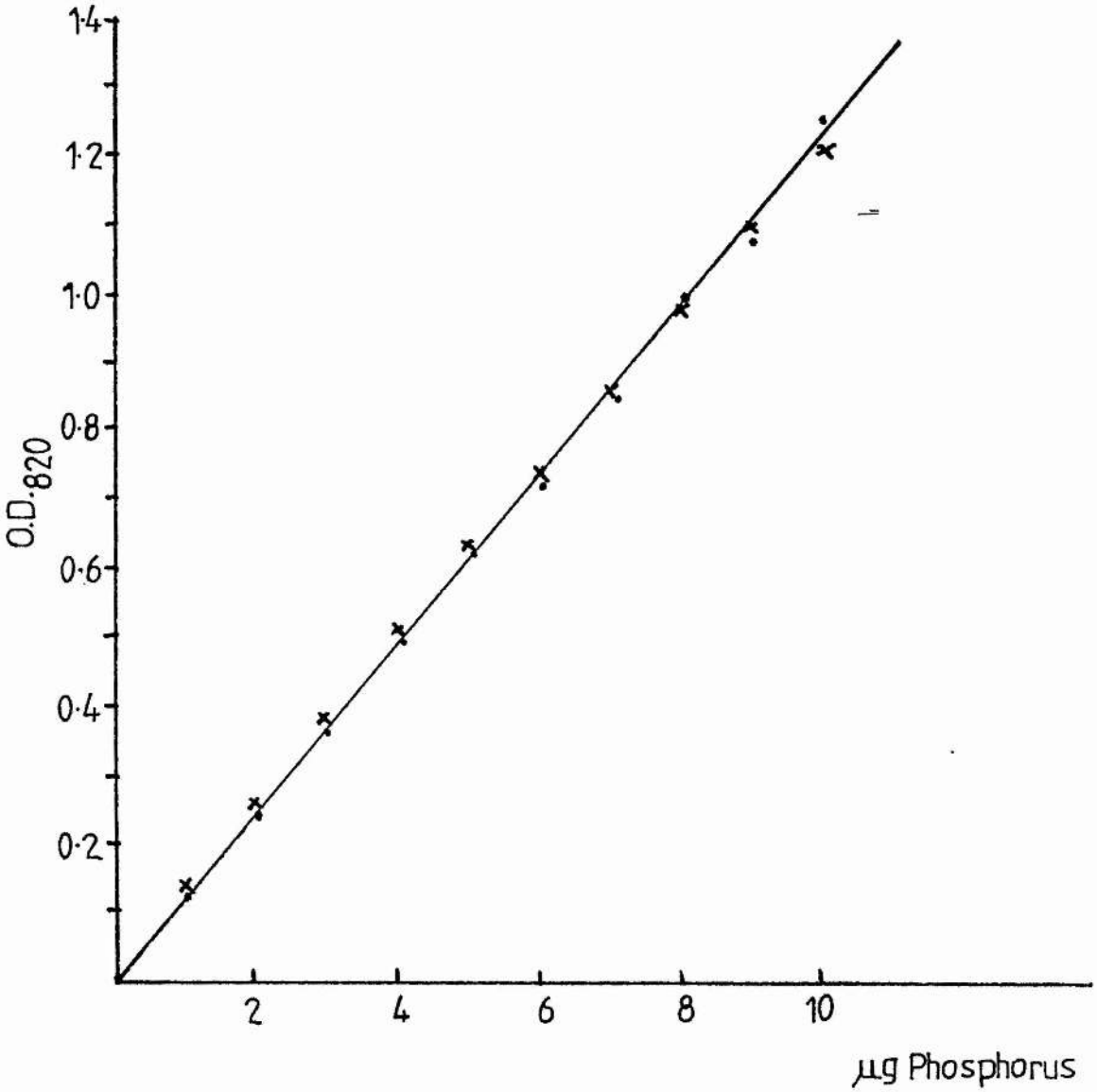


Fig. 6. Phosphorus Standard Curve.

Expt. 1 ×——×

Expt. 2 •——•

Each point is the mean of two readings.

3.6 Recovery of Lipid Phosphorus from TLC Plates

The recovery of phosphorus from TLC plates from standard SM and RBC lipid extracts was determined. The average recovery percentage in either case was over 90% and is shown in Table 10.

Table 10 Recovery of Phosphorus from Standard SM and RBC Lipid Extracts

Lipid	$\bar{X} \pm S.D$	n
Standard SM	91.2 ±4.6	7
0.3 ml packed cell lipids	98.9±5.2	6

3.7 Haemolysis Time for Ghost Preparation

10, 20, 40 and 50 minutes were used to investigate optimum time of haemolysis for ghost preparation. It was found that 50 minutes gave complete haemolysis as judged by the colour of the ghost pellet and the size of the haemoglobin spot on TLC of ghost lipids. The resultant ghosts were cream in colour and there was only a tiny haemoglobin spot on the TLC plate.

3.8 Presence of Ca^{2+} in the Buffers and the Breakdown of Phospholipids in Ghosts

The red cells used to prepare ghosts, and for phospholipase treatment of intact cells, were previously washed with 0.25mM Ca^{2+}-containing saline buffer. After the ghosts had been washed three times with hypotonic buffer, the final washing is performed in a Tris-$CaCl_2$-$MgCl_2$ buffer, before storage. During phospholipase treatment, both control ghosts and phospholipase treated ghosts were incubated for 2 hrs in the same Tris-$CaCl_2$ $MgCl_2$ buffer. After ghost preparation by this original method and incubation for 2 hrs for 37°C, phosphlipids in the control ghosts were found to be partially broken down into their lysoderivatives even though no phospholipase was added. This breakdown of phospholipids in the control ghosts must be due to the activation of endogenous phospholipase. It seems likely that this does not occur in intact cells since the intracellular Ca^{2+} concentration is maintained at a low level by the Ca^{2+}-ATPase and all intracellular phospholipases A require Ca^{2+} for activation (van den Bosch, 1980). Triplett, Wingate and Carraway (1972) have shown that the presence of $CaCl_2$ in the hemolyzing medium may result in activation of proteinase and it can be assumed a similar activation of phospholipase A would result and lead to breakdown of the phospholipid. Modifications to the procedure were therefore tried: (i) using an isotonic phosphate buffer to wash the cells instead of using Ca^{2+}-containing saline buffer, and (ii) omitting the final wash with Tris-$CaCl_2$-$MgCl_2$ buffer.

These modifications probably drastically reduce the Ca^{2+} adsorbed on the surface of the ghosts and thus minimize the subsequent activation of phospholipase A and phospholipid breakdown. Results after these modifications showed a decreasedbreakdown of phospholipids. However, as Ca^{2+} is required for the activity of the exogenous bee venom phospholipase A_2 added, the Tris buffer used for incubation had to contain Ca^{2+} in both control and enzyme-treated ghosts. It was found that, not surprisingly, some phospholipid breakdown still occured in the control ghosts (Fig. 7). Nevertheless, the phospholipid composition (combining the intact glycerophospholipids and the corresponding lyso-derivatives) was similar to that of normal intact cells (results shown in later section). This confirms the findings of Schwoch and Passow (1973) that there was no measurable loss of phospholipids from the membrane during the preparation of ghosts.

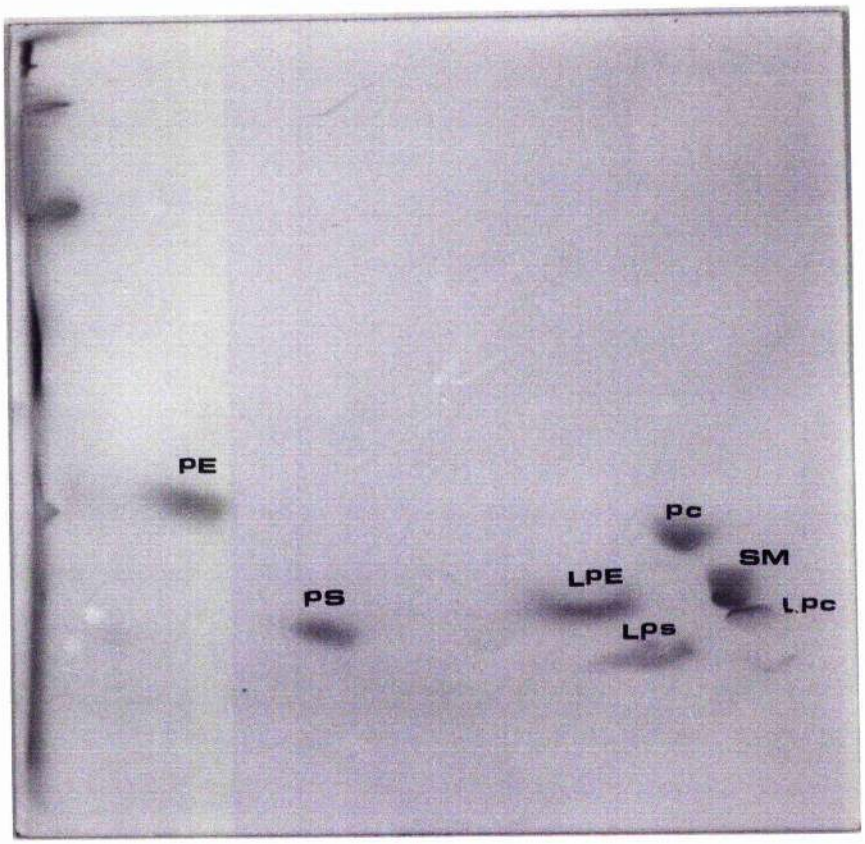


Fig. 7. Normal Ghost Lipid Extracts.
Loading: Ghosts Originated from 0.3 ml Packed Cells.

3.9 Phospholipase Treatment of Intact Red Cells and Ghosts

3.9.1 Effect of Ca^{2+} and EDTA Concentrations on Phospholipase A_2 Activity

Because 10mM Ca^{2+} was reported to be the maximum concentration for activation of phospholipase A_2 from bee venom (Shipolini, Callewaert, Cottrell, Doonan and Vernon, 1971). This concentration was used in incubations instead of 0.25mM of Ca^{2+} recommended by Zwaal and Roelofsen (1975). Final concentration of 1.6666mM EDTA Na_2 was added to stop the enzyme activity as recommended by Zwaal and Roelofsen (1975). However, the degree of haemolysis (9.4%) was found to be much higher than the acceptable 3%, and some PS degradation was found in intact cells due to this haemolysis as PS became accessible to the added enzyme. Shipolini et al. (1971) indicated that addition of both EDTA and calcium ions had a large activating effect providing that calcium ions were present in large excess. The active entity of the enzyme was a calcium-protein complex which dissociated giving inactive protein as the calcium ions formed a complex with EDTA. However, addition of EDTA and excess calcium ions led to a very large rate enhancement. It was proposed that EDTA or some EDTA-calcium complex interacted with the enzyme in such a way that the catalytic activity of the calcium-enzyme complex was increased (i.e. acting as an allosteric activator) (Shipolini et al., 1971). Cottrell (1981) offered the alternative explanation that when small amounts

of EDTA and a great excess of Ca^{2+} were present there was chelation of inhibiting metal ions that bind both to EDTA and to the enzyme more tightly than Ca^{2+} (as well as some of the Ca^{2+}), resulting in enhanced activity of the enzyme. Either of these hypotheses may explain the high degree of haemolysis % obtained in this work, if, under the initial conditions of Ca^{2+} and EDTA, addition of EDTA gave a marked stimulation of enzyme activity which may have led to membrane destabilization due to rapid production of lysophospholipids. Modifications were then made to use 0.25 mM $CaCl_2$ in the incubation buffer and increased the amount of EDTA from 1.6666 mM to 16.6666 mM (final concentration). The degree of haemolysis obtained thereafter was always less than 2%.

3.9.2 Amount of Enzyme Added and the Incubation Time

According to Zwaal et al. (1973), incubation for 1 hr with 10 U of phospholipase A_2 (from bee venom) per 0.2 ml packed cells gave 55% degradation of total PC and 9% degradation of PE. In this work, the same conditions were examined for the non-lytic degradation of the RBC, but results showed much lower degradation of PC and PE, either due to inadequate amount of enzyme added or too short an incubation time. Extending the incubation time to 2.5 hrs progressively with 10 U enzyme/0.2 ml packed cell was tried but non-ideal results still occurred. Thus increased amounts of enzyme and extended incubation times were tried under conditions that haemolysis was kept below 3%. 20 U/0.2 ml packed cell and 2 hrs incubation were finally found to be the most suitable conditions for the non-lytic maximal

degradation of RBC phospholipids. Results are shown in Table 11.

3.9.3 Haemolytic Curve

The progress of haemolysis with time under the optimal conditions just defined was determined in two experiments (Fig. 8).

Haemolysis may be taken as an index of membrane integrity. It is likely that haemolyzed cells will undergo degradation of the lipids of the inner layer of the lipid bilayer, since the enzyme can gain access to the inside of the cell. The maximum degree of haemolysis usually regarded as acceptable for asymmetry studies is 2%. 2% is likely to be a maximum error for inner leaflet phospholipid degradation since in newly haemolyzed whole cells it is likely that the phospholipids will be less accessible to the phospholipase at the cytoplasmic leaflet due to the presence of, for example, spectrin, or to resealing of membrane fragments.

3.9.4 Susceptibility of Glycerophospholipids to Phospholipase A_2 Attack in Intact Red Cells and Ghosts from Normal Controls and DMD Patients

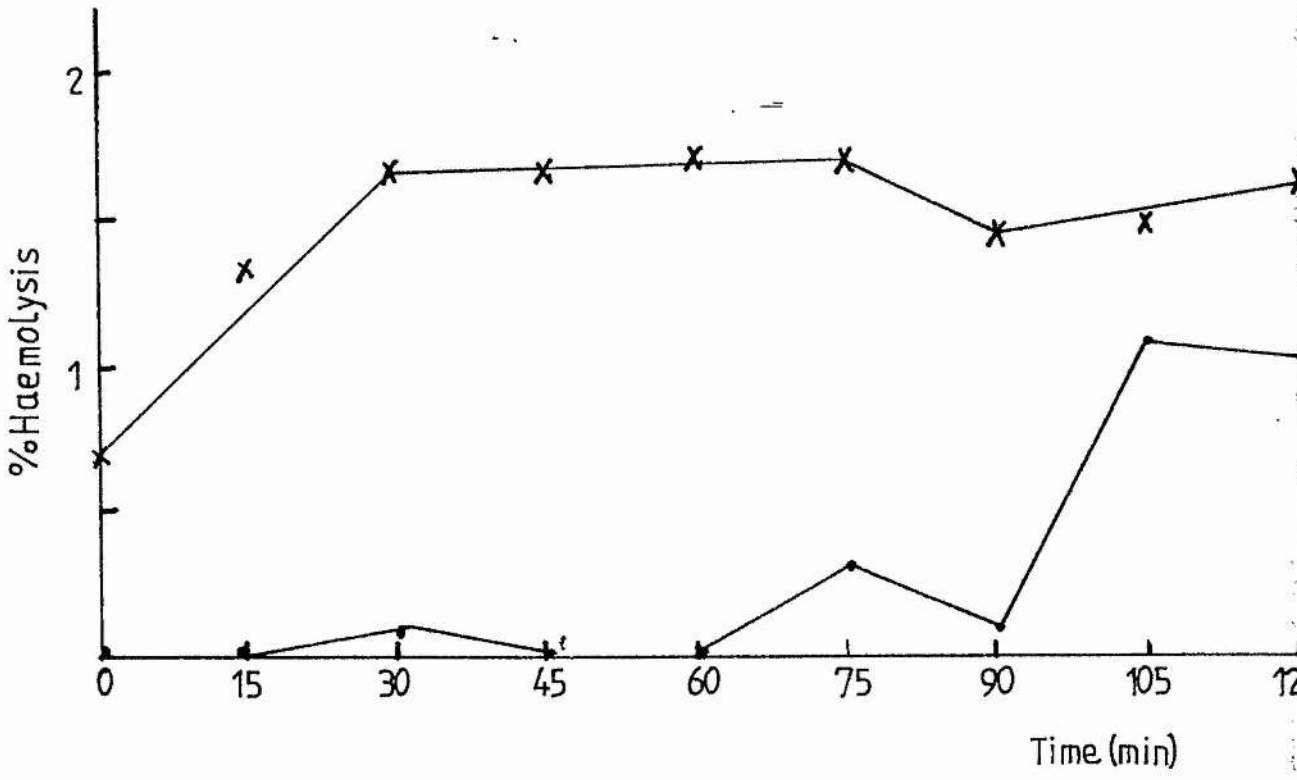
The results of non-lytic lipid degradation in normal intact red cells is shown in Table 12, a thin layer chromatogram showing lipids from enzyme-treated RBC is shown in Fig. 9.

Since SM is not a substrate for the enzyme, it is used as internal standard, degradation % of PC and PE is calculated from SM% compared to that of control (for details, see 2.5).

Table 11 Non-Lytic Degradation of Intact Red Cells

Units/0.2 ml packed cell	10	10	10	20	20 (n=5)
Incubation time (hrs)	1	2	2.5	1	2
Haemolysis %	0	1.14	0	1.33	1.24 ±0.50
PC degradation %	28.6	35.0	38.4	38.5	50.5±8.0
PS degradation %	0	0	0	0	0
PE degradation %	6.1	3.2	0	12.8	4.9±3.1

Results were expressed as x ± y; y = S.D.



Experiment A ✕——✕

Experiment B •——•

Fig. 8. % Haemolysis Following Incubation with Phospholipase A_2. (20 U/0.2 ml packed cells).

Table 12 Non-Lytic Lipid Degradation in Normal Intact Red Cells by Bee Venom Phospholipase A_2

Phospholipids	This work (n=6)	Zwaal et al., 1973	Lubin, Chin, Bastacky, Roelofsen and Van Deenen (1981)
PC	51.73±7.76	55	60±2.9
PS	0	0	0
PE	6.24±4.23	9	11±2.0
haemolysis %	1.04±0.68	not shown	not shown

Results were expressed as x ± y; y = S.D.

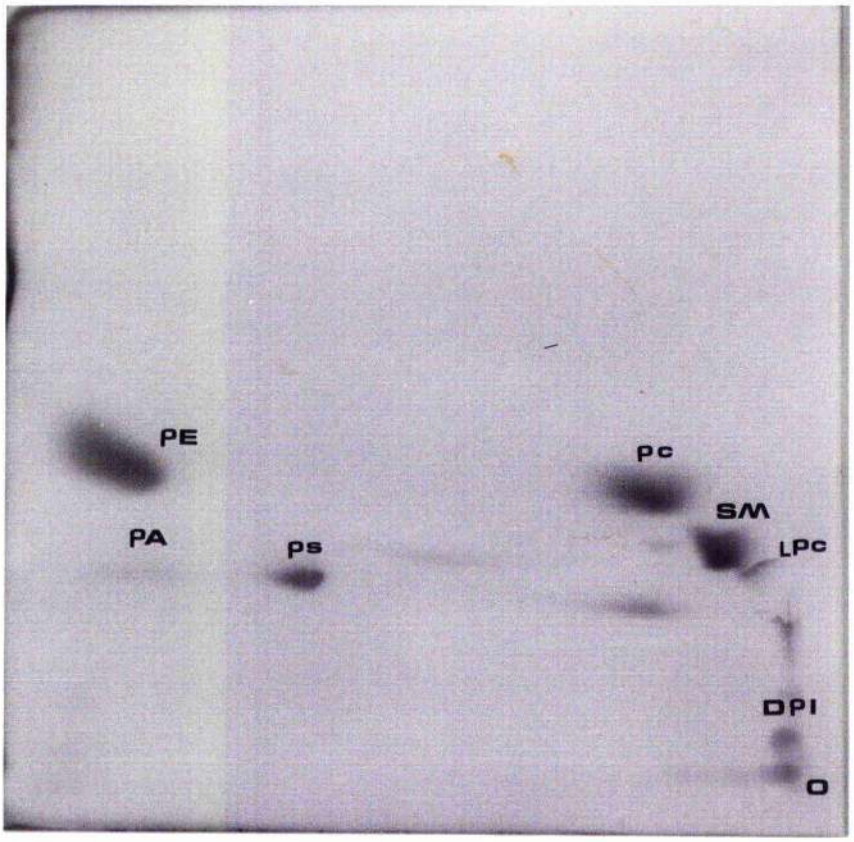


Fig. 9. Lipid Extracts of Normal RBC Incubated with Phospholipase A_2 (20 U/0.2 ml Packed Cell for 2 hrs at 37°C). Loading: Lipids from 0.3 ml Packed Red Cells.

Lipid asymmetry of human erythrocytes was first proposed by Bretscher (1972) who stated that the two choline-containing phospholipids, PC and SM, are located chiefly in the outer half of the lipid bilayer, while PS and PE are located on the inner (cytoplasmic) half of the bilayer. The erythrocyte membrane is the only biological membrane in which lipid asymmetry has been demonstrated in a convincing and conclusive way (Op den Kamp, 1979) and extensive work has been done on this cell type. Lipid asymmetry was studied by two kinds of methods. Firstly, using non-penetrating chemical reagents to label the amino-group containing phospholipids, and secondly using various kinds of phospholipases to degrade the phospholipid non-haemolytically in intact cells and unsealed ghosts.

Human RBC have few lipid aminogroups available to react with chemical agents which cannot permeate the membrane. Such reagents are designed to label free amino groups, like those in PS and PE, but not the trimethylamino groups of PC. Little PS and PE was found to be labelled in intact cells by the fluorescent reagent, disodium 4-acetamido 4'-thiocyano stilbene disulphonate (Maddy, 1964) and little PE on the exterior of RBC was able to react with a radioactive reagent, ^{35}S formylmethionyl (sulphone) methyl phosphate (Bretscher, 1972). Gordesky (1974) showed that when RBC were treated with 2,4,6-trinitrobenzene-sulfonate for 24 hrs, a maximum of 33% of the PE and none of the PS reacted with this reagent

whereas in ghosts, 95% of PE and > 50% of PS reacted in 90 mins under same condition. He concluded that all or nearly all of the PS and a minimum of 70% of PE is on the inside surface of the membrane. Another line of evidence for lipid asymmetry came from the studies with various phospholipases from natural sources. The use of a sea snake venom which possesses phospholipase A activity (Ibrahim and Thomson, 1964) indicated that SM and PC are the main phospholipid components of the exterior half of the bilayer. They found limited phospholipase action occured in the absence of cell lysis. The only detectable product they found was LPC, with no LPS or LPE. Phospholipase A_2 from Naja naja hydrolyzed 68% of the PC of the intact red cells, while sphingomyelinase from Staphylococcus aureus hydrolyzed 80-85% of the SM of the intact red cells (Verkleij, Zwaal, Roelofsen, Comfurius, Kastelijn and van Deenen, 1973). They concluded that the majority of the choline-containing phospholipids (PC and SM) and some 1/5 PE forms the outer monolayer of the membrane. The inner half of the bilayer was composed mainly of PE and PS, and a minor fraction of choline-containing phospholipid may also be present. A diagram indicating the proposed lipid asymmetry of red cells (after Verkleij et al., 1973) is shown in Fig. 10.

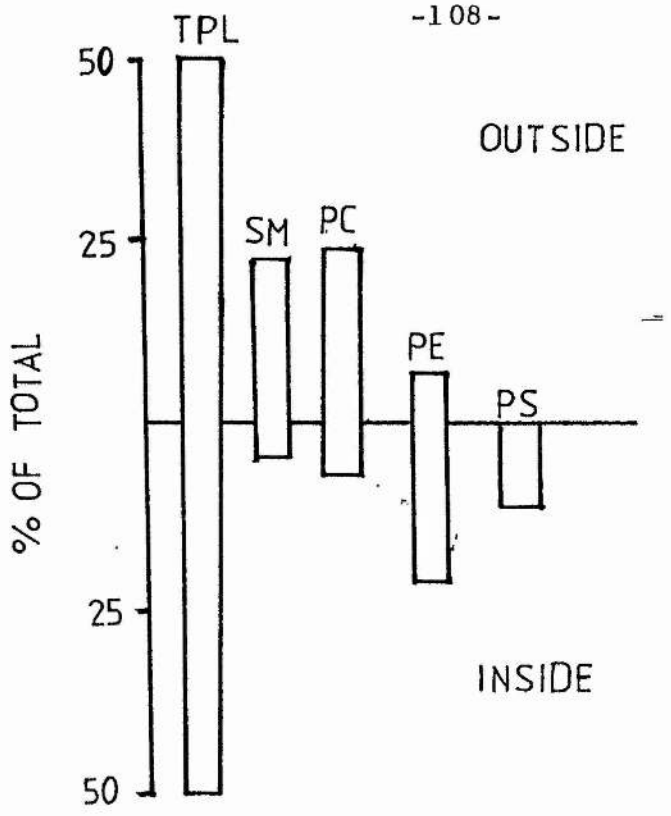


Fig. 10. Proposed Distribution of Phospholipids Between the Inner and Outer Layer of the Human RBC Membrane. Abbreviation: TPL Total Phospholipid.

The conclusions regarding lipid asymmetry from these experiments are only valid under the condition that the rate of flip-flop (exchange of the phospholipids between the two halves of the bilayer) is slow and insignificant compared with the experimental period. Flip-flop has been found to be very slow indeed, with a half-life measured in hours in intact human erythrocytes (Bloj and Zilversmit, 1976).

Phospholipase A_2 (from bee venom) has been used without further purification to study the lipid asymmetry

in human red cells and was therefore used in this work. This enzyme preferentially attacked PC in whole cells, converting over 50% of the total PC into LPC without causing lysis although the red cells became more osmotically fragile (Zwaal et al., 1973). In this work the fact that haemolysis was always less than 2% suggests strongly that the phospholipids which were susceptible to the degradative action of phospholipase A_2 were localized on the outside of the cell membrane, i.e. 55% of PC and 9% of PE were localized in the outer monolayer of the bilayer. The fatty acids and LPC produced by the action of phospholipase A_2 seemingly remain in the membrane (Roelofsen and Zwaal, 1977), organized in a bilayer configuration as observed by ^{31}P-NMR of ghosts from such enzyme-treated RBC (Van Meer, de Kruijff, Op den Kamp and Van Deenen, 1980). The importance of this stable organisation of the fatty acids and LPC, which prevents haemolysis even though a large proportion of the glycerophospholipids are degraded, is indicated by the lysis which occurs after the binding and removal of fatty acids by the addition of bovine serum albumin (Zwaal, Roelofsen and Colley, 1973). After the phospholipase A_2 (from bee venom) degradation, a stable bilayer structure is produced which consists of a monolayer of lysophospholipids and fatty acids on the outside and a monolayer of intact phospholipid on the inside (van Meer et al., 1980). It is remarkable that production of LPC in situ does not lead to cell lysis, while exogeneous LPC is a lytic agent. The

reason for this is thought to be because the fatty acids and LPC form a complex whose conformation resembles PC (Jain, van Echteld, Ramirez, de Gier, de Haas and van Deenen, 1980). In a study using 1-LPC and fatty acids in aqueous dispersion, Jain and de Haas (1981) found that acyl chain interaction between fatty acids and 1-LPC were maximized by close-packing such that the acyl chains of both molecules aligned parallel to each other and the carboxyl group was located in the vicinity of the 2-hydroxyl group of LPC. The shape and size of a functional dimer thus formed were similar but not identical to those of PC.

The phospholipid class distribution of whole RBC of DMD patients is similar to that of control (Table 13), in agreement with the results from other reports (for refs. see 1.4.1.1).

The recovery % is low sometimes because some non-lipid P-containing contaminants, for example, glycero-phosphate, may be present due to absence of washing step in the lipid extraction method.

The degradation of PC in DMD RBC was found to be higher than that of control and the difference was highly significant ($P < 0.01$, Student's t test). On average, 32% more PC was available at the outer surface of DMD RBC (Table 14 and Fig. 11). There was also no overlap between the ranges for PC degradation for DMD and controls.

Table 13 Phospholipid Class Composition of Whole RBC

(Expressed as % phosphorus (w/w))

Phospholipids	Controls (n=12)	DMD Patients (n=6)
PC	30.97±2.78	29.20±1.08
SM	26.89±2.05	29.67±2.37
PS	10.75±1.04	9.86±3.03
PE	31.21±1.79	31.27±1.19
Yield (μ mole P/ml packed cell	3.10±0.50	3.16±0.38
Recovery after TLC (%)	89.85±8.70	84.21±11.30

Table 14 Degradation of Glycerophospholipids in Whole RBC Incubated with Bee Venom Phospholipase A_2

	n	% total phospholipids degraded ($\bar{X}$ ± S.D.)			% haemolysis
		PC	PE	PS	
Controls	6	51.73±7.76	6.24±4.23	0	1.04±0.68
DMD Patients	6	68.13±6.23	6.12±4.63	0	1.18±0.99

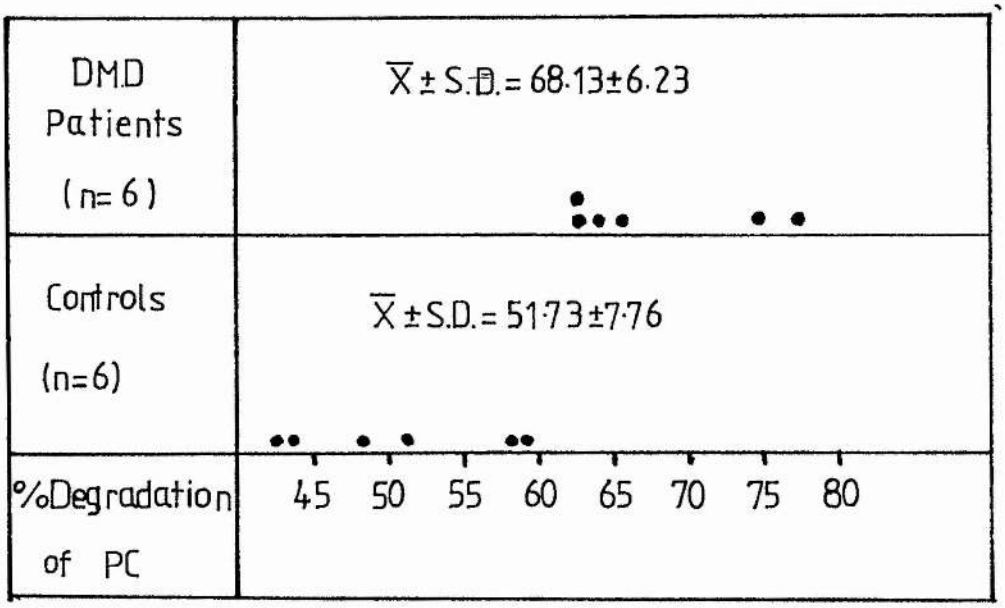


Fig. 11. Degradation of PC in Whole RBC in Controls and DMD Patients.

Thin-layer chromatograms of lipids from control and enzyme-treated DMD RBC are shown in Fig. 12 and 13.

The phospholipid class distribution of DMD ghosts were found to be similar to that of controls. (Table 15) and in both cases these correspond closely to the composition of whole cells.

When DMD and control ghosts were treated with the

enzyme, all of the glycerophospholipids underwent complete conversion to their corresponding lyso-derivatives, indicating that those phospholipids in the native membrane are fully available to bee venom phospholipase A_2 where access to both sides of the membrane is possible. Thin-layer chromatograms showing the lipids of enzyme-treated normal ghosts, DMD ghosts and enzyme-treated DMD ghosts are shown in Fig. 14, 15, 16 respectively.

Increased PC degradation at the outer surface of the membrane of DMD red cells may be interpreted in at least two ways, either (i) the distribution of PC is normal but during the course of the enzyme treatment, transbilayer translocation (flip-flop) of PC molecules, normally associated with the cytoplasmic leaflet, occurs more readily than in normal erythrocytes; or (ii) a greater proportion of the total PC is localized in the outer leaflet.

The half time of transbilayer movement of PC observed in intact human erythrocytes was 2-5 hrs and half times of the same order of magnitude were found for the transbilayer movement of aminophospholipids (PE and PS) (Bloj and Zilversmit, 1976). During the time-course of the incubation used in this work (2 hr) therefore one would not expect any significant transbilayer movement of PC in the normal RBC. If the increased degradation of PC in DMD RBC was due to the increased flip-flop of PC during the incubation, this must reflect some perturbation of organization in the DMD RBC membrane. Alterations

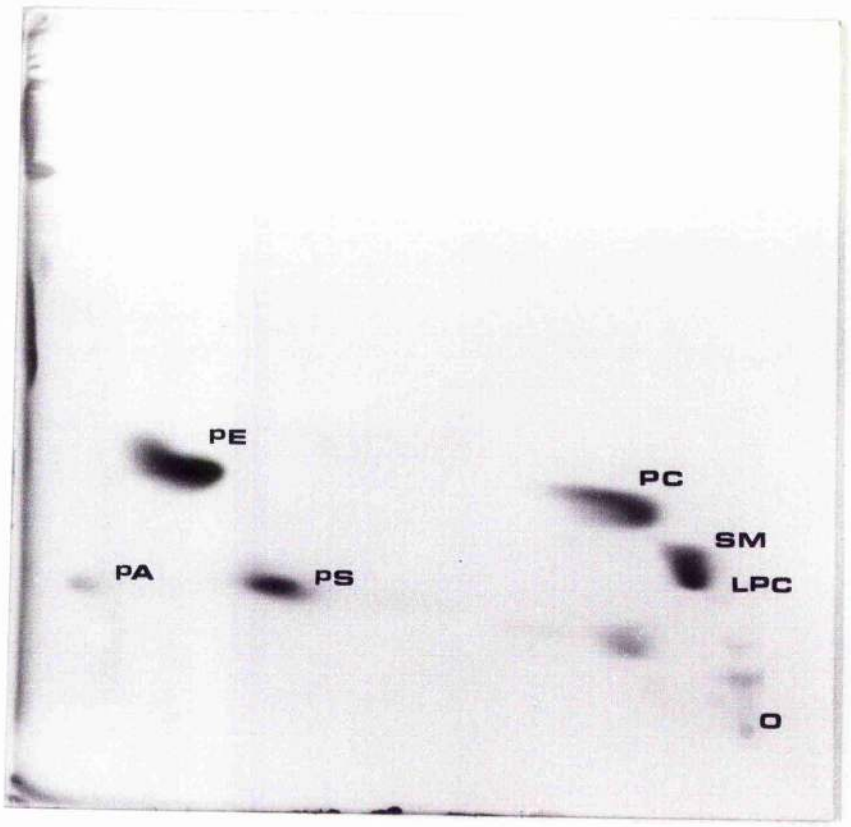


Fig. 12. Lipid Extracts of DMD RBC (Control).
Loading: Lipids from 0.3 ml Packed Cells.

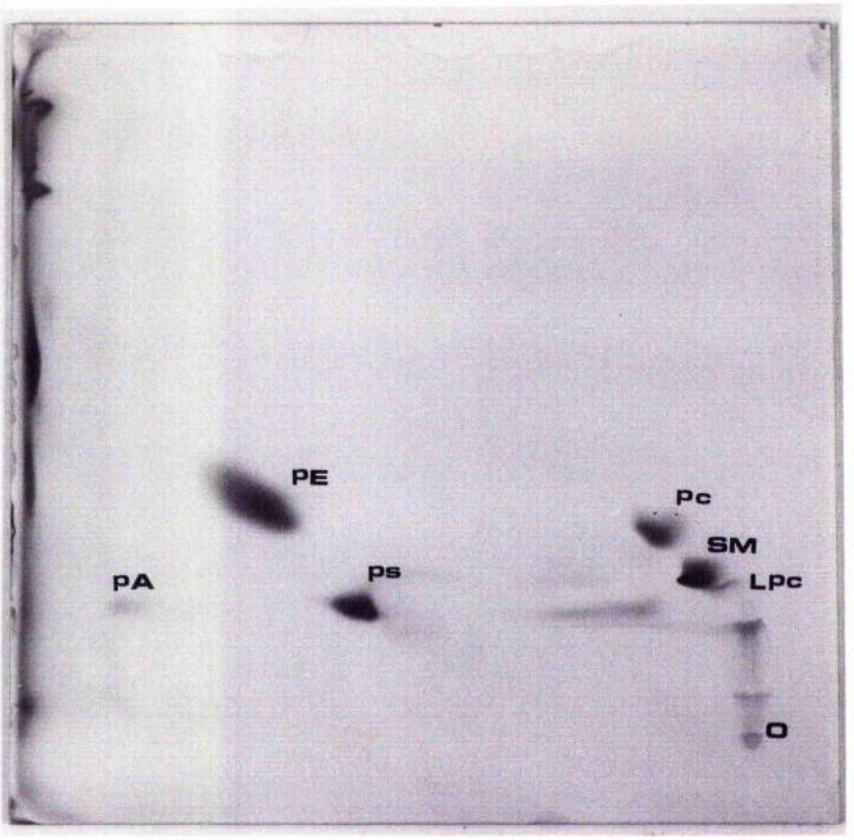


Fig. 13. Lipid Extracts of DMD RBC Incubated with Phospholipase A_2 (20 U/0.2 ml Packed Cells for 2 hrs at 37°C). Loading: Lipids from 0.3 ml Packed Red Cells.

Table 15 Phospholipid Class Distribution of DMD Ghosts and Control Ghosts (expressed as % phosphorus (w/w)).

Phospholipids	DMD Ghosts (n=5)	Control Ghosts (n=5)
PC	28.419±1.46	30.93±2.78
SM	31.265±4.69	26.464±2.90
PS	11.686±3.60	12.805±1.87
PE	27.751±0.65	29.429±1.30
Yield (μ mole lipid P/ml packed cell)	2.70 ± 0.42	2.93 ± 0.54
Recovery %	85.5 ±5.24	85.14 ±9.34

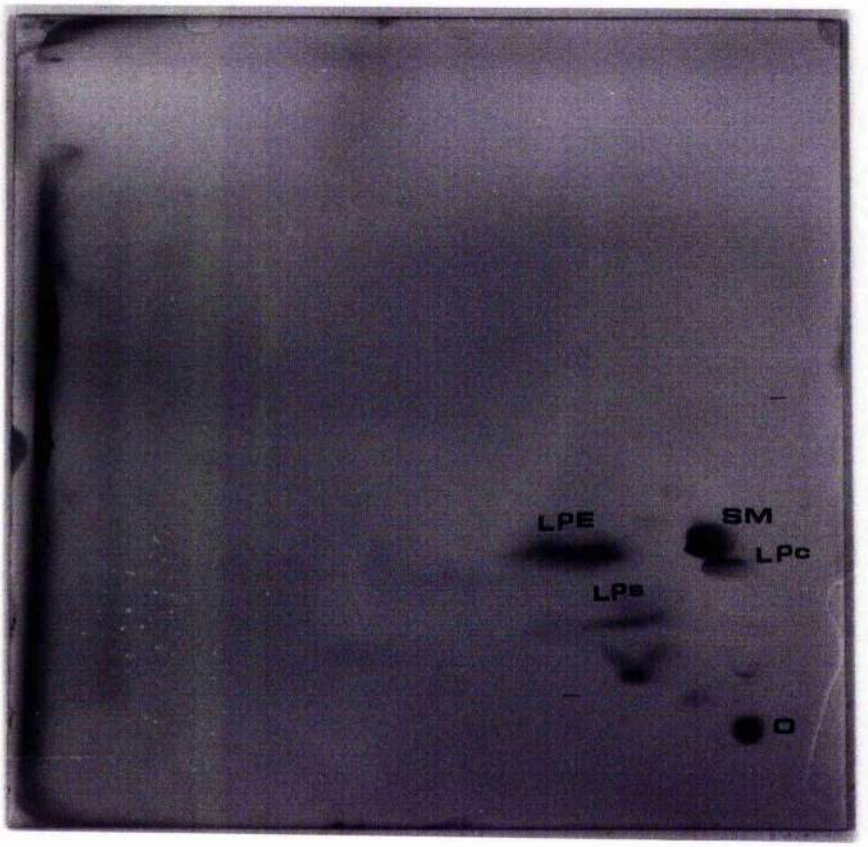


Fig. 14. Lipid Extracts from Normal Ghosts Incubated with Phospholipase A_2 (20 U/ 0.2 ml Packed Cells for 2 hrs at 37°C). Loading: Ghosts Originated from 0.3 ml Packed Cells.

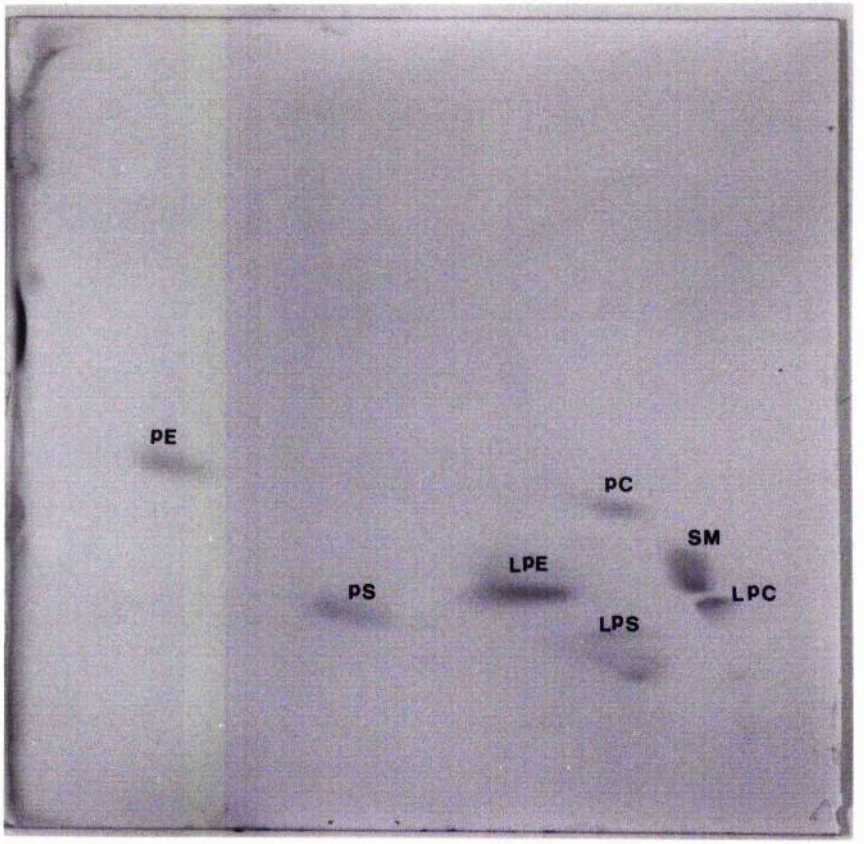


Fig. 15. Lipid Extracts from DMD Ghosts (Control). Loading: Ghosts Originated from 0.3 ml Packed Cells.

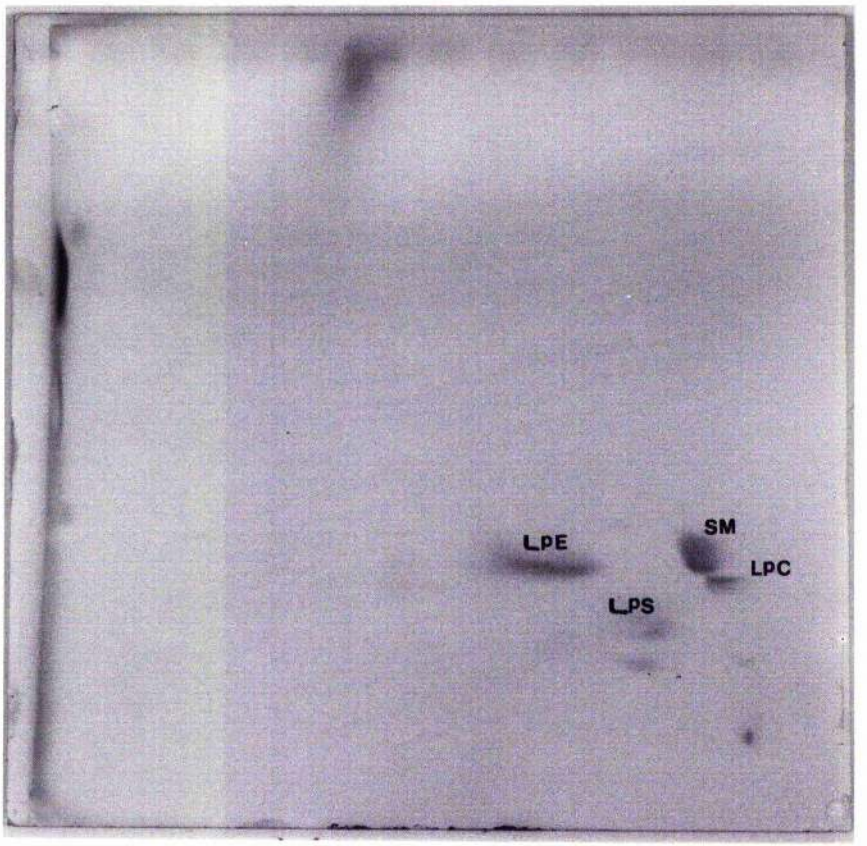


Fig. 16. Lipid Extracts from DMD Ghosts Incubated with Phospholipase A_2 (20 U/0.2 ml Packed Cell for 2 hrs at 37°C). Loading: Ghosts Originated from 0.3 ml Packed Cells.

in spectrin may be a possible cause of the increased transbilayer movement of PC. Spectrin, a component of the cytoskeleton, was found to be important in maintaining the lipid asymmetry in erythrocytes (Haest, Plasa, Kamp and Denticke, 1978). It was found that changes in lipid asymmetry may occur after the oxidation and phosphorylation state of spectrin is changed. After using SH-oxidizing agents to cross-link spectrin monomers to form oligomers, 30% of the total PS and 50% of the PE became available for hydrolysis by phospholipase A_2 (from bee venom) under non-haemolytic conditions. No PS and about 5% PE will normally be hydrolyzed in unoxidized erythrocytes, so that they suggested that a release of constraints imposed by spectrin enabled PS and PE to move from the inner to the outer lipid layer of the RBC membrane and that spectrin in the native RBC, stabilized the orientation of these phospholipids at the inner surface of the membrane. This is supported by the finding that between 19-25 PE molecules and 13-17 PS molecules were covalently linked to each spectrin dimer by bifunctional cross-linking reagents (Marchesi, 1979). Gazitt, Ohad and Loyter (1976) showed that in chick RBC, dephosphorylation of membrane proteins (including spectrin) might induce the aggregation of intramembrane particles (as seen in freeze-fracture EM) and relatively increase the availability of the free lipid bilayer to phospholipase A and trinitrobenzene sulfonic acid, i.e. more phospholipids were exposed. The effect of protein dephosphorylation might be expressed in terms of changes in the compressibility of the lipid bilayer

(Demel, Geurts van Kessel, Zwaal, Roelofsen and van Deenen, 1975) which is known to influence the susceptibility of phospholipids to phospholipases.

Franck, Roelofsen and Op Den Kamp (1982) showed a complete exchange of PC from intact RBC was possible after protein cross-linking, using specific PC exchange protein. So much evidence therefore has proved the close relationship between spectrin and lipid asymmetry. It is significant then that spectrin has been reported to be abnormal in DMD erythrocytes. Teuchiya et al. (1981) and Nagamo et al. (1980) indicated spectrin extractability was significantly reduced in DMD ghosts. Roses et al. (1975; 1976; 1977; 1979; 1981) demonstrated the phosphorylated state of spectrin in DMD RBC was normal, but found an increased phosphorylation in Band II (one of two electrophoretic bands for spectrin in SDS-PAGE) although conflicting results have also been presented. Although some of the evidence presented above seems to relate only to an increase in PE and PS transfer to the outer lipid bilayer, nevertheless, it seems likely that any changes in the cytoskeleton may influence the lipid asymmetry and that some more subtle abnormality in spectrin of DMD erythrocytes (such as phosphorylation) may bring about the increased PC degradation found in this work. In sickled RBC, in which abnormality in the spectrin-actin lattice has been shown, more PE and PS are translocated to the outer layer from the inner leaflet while PC is transferred from the outer leaflet to the inner leaflet in exchange for PE and PS (Lubin, Chin,

Bastacky and Roelofsen, 1981).

If abnormal spectrin is considered to be the cause of the increased PC degradation found in this work, an abnormal spectrin and the resultant change in lipid asymmetry may well be the cause of changes in deformability of the DMD RBC found in some laboratories. Palek and Liu (1979) proposed that the stomatocyte-discocyte-echinocyte equilibrium depends on the relationship between the surface areas of the inner and outer halves of the membrane bilayer. Since spectrin and actin form a two-dimensional network under the inner leaflet of lipid bilayer, re-arrangement could produce a decrease in surface area both of the spectrin-actin network and the closely associated inner half of the membrane lipid bilayer. The resulting modification in bilayer asymmetry could then be reflected in an echinocyte shape change. Reduced deformabilities and increased percentage of echinocytes in DMD RBC have been found in numbers of laboratories although almost the same number of contrary reports have been presented (for details, see 1.4.3.1). The significance of the altered lipid asymmetry in DMD RBC found in this work cannot be ignored in relation to the deformability and shape changes of DMD RBC. In sickle cell anaemia, an altered RBC shape (sickled) was found as the result of abnormal spectrin and altered lipid asymmetry (Rice-Evans and Dunn, 1982). In hereditary spherocytosis, reduced deformability of RBC was found to be related to the altered phosphorylation of spectrin (Rice-Evans and Dunn, 1982). From the above evidence, an altered lipid

asymmetry might also be expected, although this has not been examined.

If a greater proportion of total PC genuinely localized in the outer leaflet is the explanation for the observation in this work, then, in order to maintain a constant pressure in the outer leaflet, some other components in the outer leaflet must either be reduced or transferred to the inner leaflet. Two components can be proposed, i.e. lipids or integral protein. In the outer leaflet, SM and cholesterol are the only two lipids present in substantial amounts, besides PC. If one or both of these components are transferred preferentially to the inner leaflet to compensate for increased PC in the outer leaflet, this would be expected to alter membrane properties considerably. Further experiments, such as probing their distribution using sphingomyelinase and cholesterol oxidase, can be proposed to examine this hypothesis. Depletion of integral protein associated solely with the outer half of the bilayer, such as acetyl cholinesterase, could also counter-balance the increased pressure exerted by increased PC localized in outer leaflet. However, this enzyme has been shown to have normal activity in DMD erythrocyte (Igisu, Mawatari, Kuroiwa and Miyoshino, 1980). Whatever the compensating mechanism, such a change in lipid organization is likely to lead to altered membrane physical properties.

As more PC is located at the outer leaflet, a decreased SM/PC would be expected there. An altered

SM/PC will produce profound effects on the properties of the bilayer (Barenholz and Thompson, 1980). Shinitzky and Inbar (1976) indicated in artificial phospholipid vesiclesthat an overall decreased SM/PC was coincident with an increase in fluidity as SM normally contains more saturated fatty acids than PC. This was confirmed in sheep erythrocytes (Borochov, Zahler, Wibrandt and Shinitzky, 1977). A decreased membrane viscosity can also be expected to accompany the decreased SM/PC. In a theoretical study by Barenholz and Thompson (1980) using a mixture of egg PC and brain SM, increased apparent microviscosity was observed in all systems examined at all temperatures in the range of 0^{o}-60^{o}C with increasing content of SM. In abeta-lipoproteinemia, patients have acanthocytic RBC, with increased SM/PC as high as 1.56 (normal value is 0.64). Associated with this change was an increase in microviscosity and decreased membrane deformability (Cooper, Durocher and Lestie, 1977). In summary, a decreased SM/PC may leadto increased fluidity and decreased viscosity in the outer leaflet of the membrane of erythrocytes.

Another possible change which may result from increased PC in the outer leaflet of the lipid bilayer is a decrease in cholesterol to give a decreased ratio of cholesterol to phospholipids (Chol/PL). Decreased chol/PL has a similar effect on the membrane as does decreased SM/PC. In studies on guinea pig RBC, Kroes and Ostwald (1971) demonstrated a tighter packing of the lipid component after cholesterol loading. The addition

of cholesterol to pure phospholipid membrane enhanced the degree of order within membrane lipid and caused decreased fluidity and increased viscosity (Cooper, 1977). So that, decreased cholesterol could induce increase fluidity and decreased viscosity in the outer leaflet of membrane of erythrocytes.

Altered fluidity in DMD erythrocyte membranes was found by ESR in the polar and hydrophobic region of the lipid bilayer (for refs., see 1.4.3.3). Although some results (for example, decreased fluidity at the surface region observed by Wilkerson et al. (1978)) do not seem to coincide with the effect (increased fluidity) of the proposed decreased SM/PC or decreased chol/PL, it must be realised that the effect of changes in lipid ratios does not necessary cause a significant change in the bulk fluidity of the entire membrane but may cause critical changes within specific domains of the membrane (Cooper, 1978). Thus it is still likely that the altered PC distribution may lead to some or all of the spectroscopic abnormalities found in DMD erythrocytes.

Osmotic fragility was found to be directly proportional to the degree of fluidity of the lipid bilayer (Weed and Reed, 1966). Danieli and Marchesini (1980) and others (for details, see 1.4.3.2) showed an increased RBC osmotic fragility in DMD patients and carriers. This could therefore be the result of increased fluidity due to decreased SM/PC or chol/PL in the outer leaflet. By a direct influence on fluidity or perhaps by some allosteric mechanism, lipid composition modulates

the activity of membrane bound enzymes and the transport processeswith which they are associated (Cooper, 1977). Changes in fluidity may influence the range of motion or the potential volume available to a membrane enzyme and its substrate during the enzymic process (Cooper, 1978). It is worth recalling that ion-transport and enzymic activities in DMD RBC have been found to be altered by many laboratories; although contrary findings have also been presented (see 1.4.4.2.1 and 1.4.5.1).

Increased K^+ efflux was found in DMD RBC (Dise et al., 1977), which is consistent with decreased chol/PL, since when chol/PL decreases, the membrane permeability to several substances that enter by carrier-mediated transport, or facilitated diffusion is increased (Cooper, 1977; Kroes and Ostwald, 1971). However decreased K^+ efflux was reported by Szentistvánýi et al. (1980) and Ca^{2+} influx and efflux was found to be normal (Shoji, 1981) in DMD RBC.

Increased Ca^{2+}-Mg^{2+} ATPase activity and altered basal activity and response to ouabain by Na^+-K^+ ATPase could be explained by the abnormal lipid domain adjacent to the enzymes. Although PS and PE were considered to be the main lipids connected with the activities of ATPases (Roelofsen and Deenen, 1973), a change in fluidity may exert its effect on the enzymes via the influence on the physical state of PS and/or PE. Other changes in membrane bound enzyme activities (for details see, 1.4.5.2) could also be explained in a similar way.

Plasma membrane lipid asymmetry has been conclusively established in erythrocytes of various species and in lymphocytes. It would, of course, be of particular interest to know whether such asymmetry is also reflected in sarcolemma since the generalized membrane defect proposed as the underlying cause of DMD could well prove to be an abnormality in the normal asymmetric organization of plasma membrane lipids. If the abnormality were specifically one of PC distribution then the fact that muscle membranes contain very high PC (50% total phospholipids (Kunze, 1977)) may explain why the defect produces such severe effects in muscle rather than other tissues.

If such an abnormality in sarcolemmal phospholipid asymmetry does exist then this would almost certainly result in profound changes in the physical properties of the membrane as previously proposed for erythrocytes and consequently could account for some or all of the abnormal membrane-bound enzyme and transport functions reported (see 1.4.4.1.2, 1.4.4.2.3 and 1.4.5.2).

A noteworthy possible influence of abnormal lipid asymmetry in DMD muscle (if proved) may be on Ca^{2+} permeability. An increase in Ca^{2+} in DMD skeletal muscle was found by Maunder-Sewry et al. (1980) and Bodensteiner and Engel (1978). Ca^{2+} has received much attention because of its ability to activate various hydrolytic enzymes (ex. proteases), which may lead to muscle degeneration. Kar and Pearson (1976, 1978, 1980) have shown raised protease activity in DMD muscle. As stated above, increased fluidity of the membrane will facilitate ion transport.

It is perhaps relevant here to review the hypothesis of muscle degeneration of Wrogemann and Pena (1976) (see 1.3.2.). They proposed a sarcolemmal defect will increase the net influx of Ca^{2+} which will in turn induce functional and structural damage to mitochondria, elevated cytoplasmic Ca^{2+} levels and cell necrosis. The 'sarcolemmal defect' might be precisely a defect in lipid asymmetry which produces the various abnormalities observed in DMD muscle and particularly increased Ca^{2+} permeability leading to muscle necrosis.

Observations in this work have presented strong evidence for abnormal membrane organization in DMD. Further work is needed to explore (i) the cause of the increased PC in the outer leaflet of DMD erythrocytes, (ii) lipid asymmetry in the membrane of muscle, (iii) its relationship, if any, to the mechanism of muscle degeneration and finally (iv) a method of curing the disease.

The findings of this work may have diagnostic value if similar abnormal membrane lipid asymmetry is found in erythrocytes of carriers and affected foetuses. However the procedure would be time consuming and would probably only be worth using if the differences were as clear cut as for the patients examined in this work.

3.10 Possible Role of Plasma in Abnormal Phospholipid Asymmetry

The possibility that some or all of the 'membrane' abnormalities in non-muscle cells might be due to a circulating factor released from dystrophic/necrotic muscle has been suggested by two groups (Lloyd and Emery, 1981; Siddiqui and Pennington, 1977). This suggestion is based on the observation that the abnormal response of the red cell Na^+-K^+ ATPase to ouabain found in DMD erythrocytes can be mimicked innormal erythrocytes by incubating them with DMD plasma. To test whether such a factor might be responsible for the abnormal phospholipid organization found in this work, normal cells were incubated in the presence of normal and DMD plasma prior to phospholipase treatment in the normal way.

The composition of the four major phospholipid classes in normal red cells incubated with normal plasma and DMD plasma are similar, and not significantly different from red cells not incubated with plasma. Results are shown in Table 16.

The only difference is that both groups of incubated cells gave a lower yield of total lipid phosphorus ($P < 0.001$, Student's t test) than the red cells without incubation. The red cells incubated with plasma were subjected to five washings compared to the four washings of control red cells. The lower yield may be due to the loss of lipids from red cells in saline in glass

Table 16 Phospholipid Class Distribution of Red Cells and Those Incubated with Normal and DMD Plasma (n=6) (expressed as % phosphorus (w/w))

Phospholipid	Red cells (no incubation)	Red cells (incubated with normal plasma)	Red cells (incubated with DMD plasma)
PC	30.97±2.78	29.067±1.44	28.78±1.50
SM	26.89±2.05	24.8±1.17	25.08±1.48
PS	10.75±1.04	12.06±3.04	11.86±1.90
PE	31.21±1.79	28.21±1.60	28.37±2.80
Yield (μ moles lipid P/ml packed cell)	3.10±0.50	2.29±0.25	2.46±0.29
Recovery %	89.85±8.7	94.85±6.66	95.40±7.20

(Lovelock, 1954) and repeated saline washing may result in total phospholipid loss of up to 25-28% (Rumsby, Little, White and Tovey, 1979). However, from the analysis of classes, if loss occurs there is uniform loss of all phospholipid classes.

The susceptibility of glycerophospholipids to phospholipase A_2 incubated with DMD and normal plasma were similar, but the PC degradation of both groups was slightly higher than those from red cells without incubation. The difference between PC degradation of control red cells and red cells incubated with normal plasma was significant ($P < 0.02$, Student's t test). But the difference in degradation between both groups with incubation is insignificant. Results are shown in Table 17 and Fig. 17.

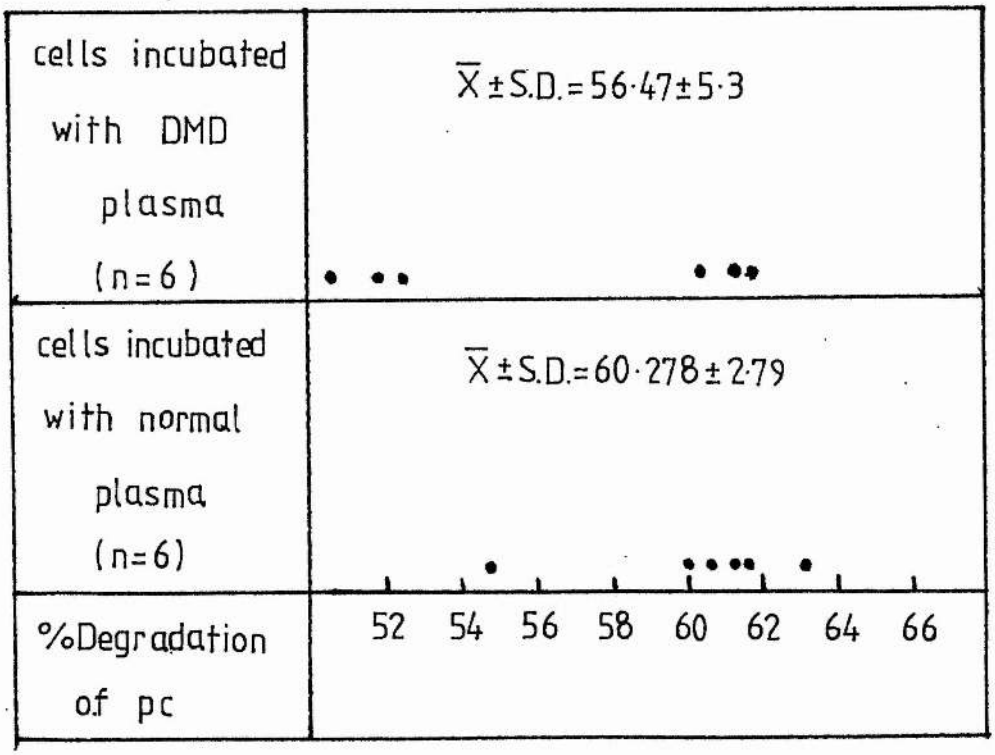


Fig. 17. PC Degradation in Whole Erythrocytes Incubated with DMD and Normal Plasma.

Table 17 Phospholipase A_2 Degradation of Phospholipids in Whole Erythrocytes Incubated with DMD and Normal Plasma

	n	% Total Phospholipid Degraded ($\bar{X}$±S.D.)			Haemolysis %
		PC	PE	PS	
Red cells without incubation	6	51.73±7.76	6.24±4.23	0	1.04±0.68
Red cells incubated with normal plasma	6	60.278±2.79	4.85±3.80	0	1.6±0.71
Red cells incubated with DMD plasma	6	56.47±5.30	4.57±3.80	0	1.15±1.10

The total time course of incubation (with plasma and then with enzyme) of plasma-treated red cells was 4 hrs compared with 2 hrs for red cells without incubation. It is possible that the extra incubation time in the presence of plasma depletes the cells of ATP which affects the phosphorylation state of the cytoskeleton and, in turn, allows more PC to be degraded at the outer surface.

These results suggest that a plasma factor, if indeed one exists, is not responsible for the increased degradation of PC in DMD erythrocytes treated with phospholipase A_2.

CHAPTER II MULTIPLE SCLEROSIS

1. INTRODUCTION

1.1 Epidemiology and General Features

Multiple Sclerosis (MS) is the commonest neurological disease affecting young adults in temperate regions (Millar, 1977). It shows regions of high prevalence (30 to 80 cases per 100,000 population) in northern Europe between 65^{o} and 45^{o} north latitude and in the northern United States and southern Canada, as well as in southern Australia and New Zealand (McFarlin and McFarland, 1982). Blacks of African origin and orientals, including Eskimos, red Indians and peoples of the Indian subcontinent rarely have this disease (Acheson, 1977).

The risk of first developing symptoms rises steeply with age from early adolescence, reaches a peak in the early thirties and falls away until it becomes trivial in the sixth decade. MS attacks women more frequently than men and on average at a slightly earlier age (Acheson, 1977).

There is increasing evidence that part, if not all, of the global variation in the frequency of the disease is associated with HLA haplotype distribution. The histocompatibility antigens HLA-A_3 and HLA-B_7 occur substantially more commonly in patients with MS than in persons from the same area without the disease. This phenomenon may be secondary to an increased frequency of another factor known as the mixed lymphocyte culture determinant HLA-DW_2. These findings give indirect evidence of the existence of a genetic factor associated with susceptibility to MS (Acheson, 1977).

An environmental factor has also been proposed as part, if not all, of the explanation of the global distribution of MS. This proposal is supported by the prevalence of this disease in immigrants. Migrants from areas of high risk to areas of low risk acquire the lower risk, provided that migration occurs before the age of 15. Conversely, people migrating in childhood from an area of low risk to an area of high risk have an increased risk (McFarlin and McFarland, 1982). So now it is generally accepted that the disease is caused by a combination of genetic predisposition and environmental factor(s). The possible nature of these factors is discussed in the following sections.

1.2 Symptoms

MS is a demyelinating disease characterized by the occurrence of lesions (plaques) in the central nervous system (CNS).

Clinically, MS is highly variable. Sensory, visual and motor dysfunction are common in patients with MS. In approximately 60% of patients, the disease is initially manifested by exacerbations and remissions. In the early stages of disease the remissions are usually associated with complete or nearly complete return of normal neurologic function, but with each remission there is less improvement and greater neurologic dysfunction. The disease enters a chronic phase and becomes progressively worse over the years (McFarlin and McFarland, 1982).

1.2.1 The Plaques

The macroscopic lesions range from 1 mm to 4 cm, scattered throughout the white matter. They are known as plaques. New lesions are pink, whereas older ones are grey. Microscopically, the characteristic feature is the breakdown of the myelin sheath, with relative sparing of axons. In new lesions products from the breakdown of myelin, largely lipids (esterified cholesterol), are found free and in macrophages (McFarlin and McFarland, 1982). Active plaques show glial proliferation, vascularization, lipid products of the breakdown of myelin, perivenular cuffing and a shelving or indefinite edge to the surrounding myelin (Adams, 1977). The active plaque also contains macrophages, lymphocytes and plasma cells (McFarlin and McFarland, 1982).

Analysis of the plaque tissue shows replacement of typical myelin proteins and lipids by glial fibrillar protein and cholesteryl ester (Davision, 1978).

Oligodendroglia, the myelin-producing cells, are reduced in number and frequently absent, at least in relatively old lesions (Prineas and Connell, 1978). But it is suggested that the primary site of the pathology is the myelin membrane rather than the oligodendroglia (McFarlin and McFarland, 1982).

1.2.2 Distributions of lesions

There are two kinds of lesions in MS. One kind extends along the course of a vein while the second kind is a centrifugal extension from the edge of a plaque. The plaques have a particular preference to follow the contours of the third and fourth ventricles. Histological study of lesions shows a small vein in the centre of nearly all plaques, except the periventricular sort (Adams, 1977).

1.2.3 Progression of lesions

Perivenular cuffs in normal or near-normal myelin may represent the initial lesion. Coalescence of such small lesions along the course (long axis) of a vein or venule may be the initial event in the formation of a plaque. An established plaque extends at its periphery rather than in the long axis of a vein. The peripheral extension may in part depend upon the degradation of myelin by lysosomal enzymes (Adams, 1977).

1.2.4 Remyelination

Some lesions may undergo a degree of remyelination in that a few lamellae of new myelin are formed (McDonald, 1974). The presence of abnormally short internodes is characteristic of remyelination in the CNS. At least part of the remyelination and repair in MS appears to depend on the ingrowth of Schwann cells from peripheral nerves and the formation of peripheral-type myelin by such cells (Feigin and Ogata, 1971).

1.3 Diagnosis

Although there is no laboratory test that can be used to diagnose MS, changes in physiological condition may be helpful.

1.3.1 Physiological tests

Investigation of visual, auditory, and somatic sensory pathways can provide evidence of lesions in the respective regions of the nervous system (McDonald and Halliday, 1977).

The following is a summary of the usually used physiological tests in the diagnosis of MS.

(a) Visually Evoked Potentials (VEP)

When light falls on the retina, a volley of impulses is set up which is transmitted through the optic pathways to the occipital cortex, where a response is evoked which can be recorded with scalp electrodes, using standard averaging techniques. MS patients may show a delay in the VEP. It is a sensitive test (McDonald and Halliday, 1977).

(b) Auditory Evoked Potentials

A high incidence of abnormality in the brain-stem components of the potential evoked by auditory stimulation has been reported by Robinson and Rudge (1975). Changes in amplitude or delayed responses or both have been found in most of the MS patients (McDonald and Halliday, 1977).

(c) Blink Reflex

Most MS patients show delayed blink reflex recorded by a surface electrode over the orbiculoris oculi muscle after electrical stimulation of the supra-orbital nerve (Kimura, 1975).

(d) Electro-oculography

Disorders of eye movement that are not obvious on physical examination may be detected by electro-oculography. (McDonald and Halliday, 1977).

(e) Electrospinogram

Electrical stimulation of the median nerve wrist evokes a response in the cervical cord which can be detected with skin electrodes and standard averaging methods. MS patients will give an abnormal electrospinogram (Small, Beachamp and Matthews, 1976).

(f) Somatosensory Evoked Response

Abnormalities in the lemniscal system can be detected by recording the somatosensory evoked response. Simultaneous recording of this response and the electrospinogram may permit a distinction between spinal cord and intraranial lesions (Baker, Larson, Sances and White, 1968).

Evoked potential deficits provide evidence of abnormality in the system mediating a response but are not specific to any particular disease process, and MS is not the only central lesion which will alter evoked potentials. Evoked potential abnormalities provide a very sensitive index of central lesions but are not diagnostic of MS or of any other specific disease process. The interpretation of the significance of an abnormal evoked potential depends on the clinical context in which it is found (McDonald and Halliday, 1977).

1.3.2 Electrophoresis tests and linoleic acid inhibition

1.3.2.1 Red cells

MS red cells showed diminished electrophoretic mobility in the presence of linoleic or arachidonic acids (Field and Joyce, 1982; Seaman, Swank, Tamblyn and Zukoski, 1979) whilst RBC from normal or other neurological disease subjects showed increased mobility under these conditions. Field and Joyce (1982) suggested that all electrophoretic mobility characteristics of RBC were conditioned by the surrounding plasma, and this did not depend upon a chemical interaction between plasma constituents and RBC membranes but was an immediate adsorption phenomenen. However this test is also the subject of contrary reports (Cuypers and Reddemann, 1980).

1.3.2.2 Lymphocytes

Lymphocytes from patients with MS are much more susceptible to the inhibitory activity of linoleic acid on their electrophoretic mobility. MS lymphocytes gave 91% inhibition while lymphocytes from normal persons showed only 57% inhibition (Field, Shenton and Joyce, 1974). They suggested that the abnormal handling of unsaturated fatty acids was a constant feature of the disease. But conflicting results have been reported, therefore these tests do not distinguish reliably between MS patients and normal subjects (Jenssen, Köhlar, Günther and Meyer-Rieneker, 1974).

1.4 Theories for the Pathogenesis of MS

1.4.1 Embolism

James (1982) proposed a subacute embolism may be the cause of MS. Fat may lodge in the microcirculation of the nervous system and cause distal perivenous oedema with the loss of myelin from axons. The main evidence to support this hypothesis is the finding that most of the small acutely demyelinated areas surround a capillary or, more usually, a small vein. In addition he drew attention to the similarity of the neurological symptoms observed in MS patients and divers suffering from nitrogen emboli resulting from too rapid decompression. As vascular damage and gliosis are commonly found outside plaques, they might be the primary event in MS and not secondary to the release of any toxic products of demyelination or to an immunological attack on myelin.

The proposed events and formation of plaques in MS are summarized in the following figures.

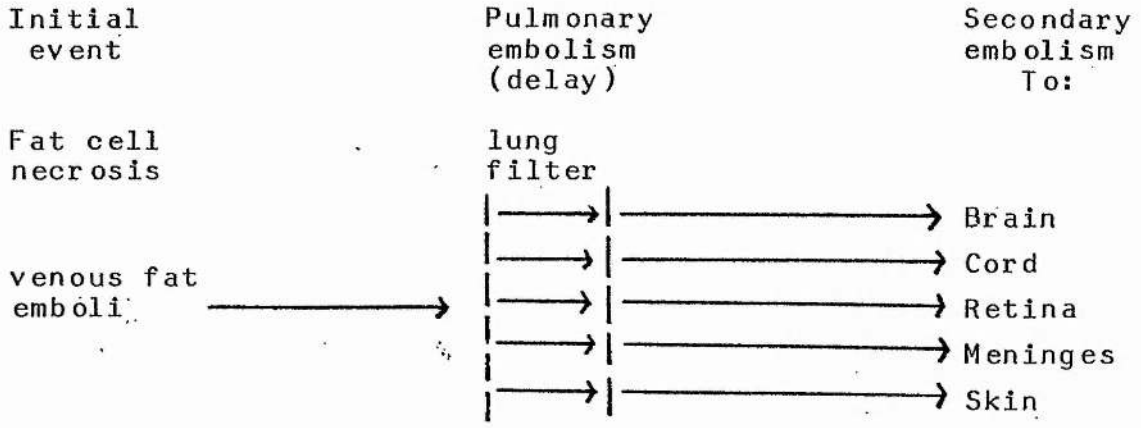


Fig. 18. Mechanism by which subacute fat embolism causes MS (after James, 1982).

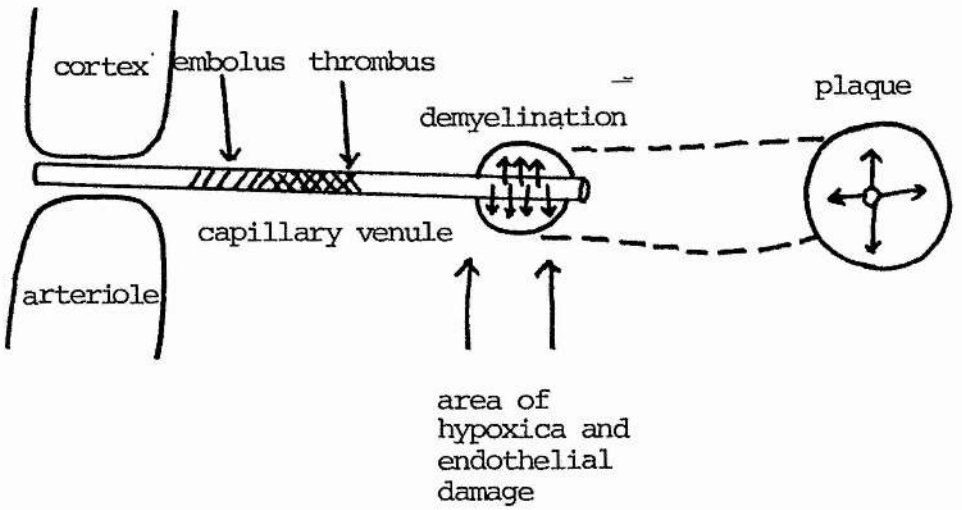


Fig. 19. Theoretical reconstitution of plaque formation in MS (after James, 1982; based on Dow and Berglund, 1942).

James (1982) suggested that depot fat is the major source of embolic fat in acute fat embolism. The properties of depot fat may contribute to some features of the disease. Depot fat may be hydrolyzed by endothelial lipases to release toxic fatty acids, and it may also contain cellular debris. The spectrum of fatty acids released depends on the composition of body fat which in turn is a reflection of the diet.

He also suggested hyperbaric oxygen treatment as an effective method to treat MS patients, as it had been used on animals with experimental allergic encephalomyelitis (the experimental-model for MS) and produced good results (Prockop and Grasso, 1978). This hypothesis has been criticized by several clinicians including Adams and High (1982), who have not detected any fat embolism in their MS tissue-bank which has CNS material from 104 cases. Also this hypothesis does not explain the immunological findings in MS.

(Blood samples from MS patients before and after hyperbaric treatment were studied in this work to compare their activities of peroxidation protective enzymes).

1.4.2 Virus infection in CNS and impaired immune response (autoimmune response)

Several lines of evidence lead to the suggestion that MS is a virus induced immune disease, i.e. a combination of environmental factor (virus infection) and impaired immune response (genetic factor).

Patients with MS were found to have had more childhood infections, indicating there had been problems in controlling various types of childhood infections (Lamouneux, Giard, Jolicoeur, Toughlian and Desrosiers, 1976). Serum of MS patients show increased antibody levels, part of which was directed against the brain itself (Ryberg, 1976). Whilst a small proportion reacted with various myxoviruses (particularly measles) (Davison, 1978; Lamoureux et al., 1976; Adams and Imagana, 1962; McFarlin and McFarland, 1982).

However the antibodies against many naturally occuring antigens (i.e. other viruses) were decreased in serum of MS patients (Lamoureux et al., 1976).

Most MS patients contained oligoclonal IgG in their CSF, a small part of this IgG was measles virus specific (Fraser, 1977). Measles-virus genome has been found in the brains of some MS patients by in situ hybridization (Haase, Ventura, Gibbs and Tourtellotte, 1981), even though viral particles can not be visualized (McFarlin and McFarland, 1982).

The whole idea is summarized by the hypothesis of Simon (1982), which was based upon experiences with animal model systems of virus-induced disease.

He proposed that a virus can act as the initiator of an auto-reactive immunopathology. Several ubiquitous RNA and DNA virus, e.g. myxoviruses, may be involved in the pathogenesis of MS. Their genome codes for peptides appearing on the plasma membrane of the host cell as neo-antigens. The virus initially attacks the mesodermal cells of the CNS, from which it can spread to neuroectoderm.

A genetically determined deficiency of the immune system then permits the persistant infection by the virus. Virus-specific antigen and/or the combination of virus- and host-specific antigens will change the antigen pattern of the host-cell membrane. Pathological cellular and/or humoral immune and autoimmune reactions preferentially affect those infected cells and their processes since those cells, carrying the neo-antigens and/or altered self-antigens, are more sensitive to immunological inquiry than noninfected cells. A structure with a slow turnover may be

particularly susceptible;such a structure is myelin. Immunopathology and cytotoxic virus action can damage the myelin sheaths or the oligodendrocytes and lead to demyelination.

This hypothesis can explain the immunological findings (which will be discussed in more detail in a later section) in MS and the pathology, duration and course of MS are all compatible with phenomena seen in various virus infections. More work is needed to confirm this hypothesis, especially the isolation and characterization of the viruses involved.

1.4.3 Fatty acids

This theory proposed a dietary deficiency in unsaturated fatty acids (PUFA) or an inborn error of fatty acid metabolism is the main factor in the pathogeneiss of MS, but does not exclude the possible associationwith the attack of a pathogenic agent, e.g. virus, and an autoimmune response. Swank (1953) suggested that the high prevalence of MS in the more northern countries was due to the high saturated fat intake (relatively deficient in PUFA) of the general population in these areas.

An inherited or acquired failure of fat transport in the blood of MS patients would leadto intravasal aggregation of red cells and platelets, and would bring about perivascular demyelination by reduction of oxygen availability in brain tissue and, by an increased permeability of the microcirculation of the brain, allow surface active materials (probably virus) to invade brain tissue and unwrap myelin sheaths (Swank, 1970). A predominance of saturated and mono-

unsaturated fatty acids might also bring about an increased rigidity of CNS membranes, rendering them more susceptible to the attack of a pathogenic agent (Alter, Yamoor and Harshe, 1974). Eylar (1972) suggested that a virus infecting the CNS might switch from its normal behaviour of lipid incorporation to increased incorporation of CNS protein into its envelope. Because of its large size and lipoprotein nature, this complex would be highly immunogenic and cause autoimmunization.

Lipid changes in various tissue, e.g. CNS, serum, red cells and lymphocytes will be discussed in more detail in the next section.

1.5 Biochemical Findings

1.5.1 Lipids

1.5.1.1 RBC and platelets

Phospholipid class distribution was found to be normal in RBC from patients with MS (Homa, Belin, Smith, Monro and Zilkha, 1980). The most consistent finding relating to the fatty acid composition of red cells from MS patients was a significantly lower level of linoleic acid (18:2) (Kurautsin-Mills, Sainji, Moscarello and Boggs, 1982; Homa et al., 1980; Gul, Smith, Thompson, Payling-Wright and Zilkha, 1970) and there was a highly significant correlation between serum linoleate and red cell linoleate (Gul et al., 1970). A similar finding was reported in platelet (Gul et al., 1970).

Kurantsin-Mills et al., (1982) found that the lower level of linoleic acid was more prominent in in-patients than out-patients, and suggested that this phenomenon might be related to the course of the disease. In healthy controls an inverse correlation was found between the percentage of linoleate and arachidonate, but in MS patients, such an inverse correlation was not found, even though the mean level of arachidonate was normal (Homa et al., 1980). They suggested that there was an abnormality in red cells of MS patients with regard to the regulation of the relative amounts of unsaturated fatty acid. In a later report by Homa (1981) an increase in lignoceric acid in the SM fraction of red cells was demonstrated but its significance for the pathogenesis of the disease is unclear.

1.5.1.2 Lymphocytes

Much less work has been done on the fatty acid components of lymphocytes from MS patients. The total fatty acid content of lymphocytes is reduced in MS (Thompson, 1975). Linoleate levels in lymphocytes obtained from MS patients have been found to be significantly below the normal level (Tsang, Belin, Monro, Smith, Thompson and Zilkha, 1976), probably due to decreased linoleate incorporation reported by Thompson (1975). A good correlation was found between the linoleate level of the plasma non-esterified fatty acids and that in the lymphocytes (Smith and Thompson, 1977).

1.5.1.3 Serum

Linoleate was found to be significantly decreased in MS serum (Baker, Thompson and Zilkha, 1964; Belin, Pettet, Smith, Thompson and Zilkha, 1971; Rosnowska, Piesio and Cendrowski, 1979; Sanders, Thompson, Wright and Zilkha, 1968; Vymazal, Ticky and Vonkova, 1977) and the degree of decrease seemed correlated with the deterioration in the patients condition (Baker et al., 1964). Normal seral linoleic acid in MS was also reported by several laboratories (Karlsson, Alling and Svennerholm, 1971; Shukla and Calusen, 1978; Tuna, Logothetis and Kammereck, 1963; Cumings, Shortman and Skulic, 1965). One group's findings (Cumings et al., 1965) of a normal seral linoleic acid level were doubtful as they used non-fasting serum samples for the experiment and the number of patients used was small, and as minimal clinical details have been given as to the state or extent of the disease it is hard to evaluate the results (Smith and Thompson, 1977).

Triene and tetraene fatty acids have been reported to be normal (Shukla and Clausen, 1978) and reduced (especially linolenic and arachidonic acid) (Rosnowska et al., 1979). On the other hand, saturated and monoenoic fatty acids of cholesterol esters were found to be increased (Vyma et al., 1977) while the level of triglyceride, cholesterol, phospholipid and their fatty acid pattern were normal according to another laboratory (Karlsson et al., 1971).

In summary, linoleate was found to be decreased in red cells, platelet, lymphocytes and serum and its decrease seems correlated with the progress of the disease. This phenomenon has led to the suggestion that the absorption or the metabolism of linoleate of MS patient may be defective. Supplementation with linoleate in the diet has been tried. But the rise in linoleate and fall in oleate after the treatment were the same in control and MS patients, proving that there was no defect in absorption from intestinal lumen in the patients (Belin et al, 1971). These authors suggested that when the amount of available linoleate increased dramatically, the related metabolic pathway may be functioning normally, whereas in the absence of these large amounts of linoleate an alternative metabolic pathway was in operation which was defective in MS. However, no finding of a defective linoleate metabolic pathway has been reported so far.

1.5.1.4 Brain

Very many reports about the lipid and fatty acid profile of the normal-looking white matter and myelin and of plaques

from MS have been published. They are summarized in the following tables (Tables 18 and 19).

No consistent changes in lipids in normal-looking white matter can be found, except perhaps increased cholesterol esters which are known to be myelin-breakdown products and formation of cholesterol esters is therefore a likely initial lipid change before true myelin- breakdown. Research on lipids in normal-looking white matter is complicated by the overlooking of minute lesions which are perhaps the reason for conflicting results reported here.

Fewer reports are available concerning lipid composition of plaques. This could be due to availability of material but also because study of normal-looking white matter can give more information about the initial changes resulting in the lesions rather than examining the already deteriorating parts of the brain. In plaques there was an increase in water content and an extensive loss of all three classes of lipids - cerebroside, sulphatide and total phospholipid (Yanigihara and Cumings, 1969; Einstein, Dalal and Csejtey, 1970). PE in plaques was decreased, and was balanced by increased amounts of PC (Cuznen and Davison, 1979). Ganglioside content of plaques was increased (Yu, Ledeen and Eng, 1974) and showed many changes in composition from the normal white matter. The major myelin ganglioside, GM_1, was decreased in plaques and there was complete absence of sialosylgalactosyl ceramide (G_7) (Cuznez and Davison, 1979). As found in normal looking white matter and myelin, cholesterol esters were found to be increased in plaques (Cuznez and Davison, 1973). This suggests that the initial

Table 18 Lipids in Normal-Looking White Matter and Myelin

Lipids	Results	References
total lipids	normal	Guzner and Davison (1973); Gerstl, Tavaststjerna, Hayman and Bond (1967)
	decreased	Winterfeld and Debuch (1977)
total phospholipids	normal	Fewster and Hirono (1976); Winterfeld and Debuch (1977)
total cholesterol	normal	Fewster and Hirono (1976);
	decreased	Winterfeld and Debuch (1977); Cumings and Goodwin (1968)
	increased	Shah and Johnson (1980)
PC	normal	Fewster and Hirono (1976); Winterfeld and Debuch (1977)
	increased	Cumings and Goodwin (1968)
PS	normal	Einstein, Dalal and Csejtey (1970)
	decreased	Winterfeld and Debuch (1977); Clausen and Hansen (1970)
	increased	Davison and Wajda (1962)
PE	normal	Winterfeld and Debuch (1977); Clausen and Hansen (1970)
	decreased	Cumings and Goodwin (1968); Gerstl, Tavaststjerna, Hayman, Eng and Smith (1965)
PI	decreased	Clausen and Hansen (1970)
ethanolamine plasmalogen	decreased	Gerstl et al. (1965); Winterfeld and Debuch (1977)
plasmalogen	normal	Fewster and Hirono (1976); Suzuki, Kamoshita, Eto, Tourtellotte and Gonatas (1973)
	decreased	Gerstl, Tavaststjerna, Hayman, Smith and Eng (1963); Davison and Wajda (1962)

Table 18 (Continued)

Lipids	Results	References
cerebroside	normal	Eng, Chao, Gerstl, Pratt and Tavaststjerna(1968)
	decreased	Gerstl, Eng, Tavaststjerna, Smith and Kruse (1970); Cumings and Goodwin (1968)
	increased	Clausen and Hansen (1970)
cholesterol esters	increased	Shah and Johnson (1980); Cuzner and Davison (1973); Davidson and Wajda (1962)
SM	normal	Fewster and Hirono (1976); Cumings and Goodwin (1968)
	decreased	Davison and Wajda (1982)
sulfatide	normal	Fewster and Hirono (1976); Gerstl, Eng, Hayman, Tavaststjerna and Bond (1967)
	decreased	Clausen and Hansen (1970)
	increased	Cumings and Goodwin (1968)

Table 19 Fatty acids (FA) in Normal-Looking White Matter and Myelin

Fatty acids	Results	References
FA and aldehyde composition of cholesterol esters, phosphoglycerides and sphingolipid	normal	Fewster and Hirono (1976); Gerstl et al. (1970)
$C_{16:1}$ of total lipid fraction	increased	Clausen and Hansen (1970); Boggs and Mascarello (1980)
	decreased	Thompson (1972)
PC saturation	normal	Arnetoli, Pazzagli and Amaducci (1969)
	decreased	Clausen and Hansen (1970)
	increased	Baker, Thompson and Zilkha (1963)
$C_{18:0}$, $C_{18:1}$ in PC	decreased	Winterfeld and Debuch (1977)
$C_{20:4}$ in PC	decreased	Clausen and Hansen (1970)
	normal	Arnetoli et al. (1969)
$C_{16:0}$ in PC	increased	Winterfeld and Debuch (1977)
PE unsaturation	increased	Clausen and Hansen (1970)
$C_{18:1}$ in PE	decreased	Arnetoli et al. (1969); Winterfeld and Debuch (1977)
$C_{20:1}$ in PE	decreased	Gerstl et al. (1970)
	normal	Fewster and Hirono (1976)
polyenoic acid in PE	increased	Alling, Vanier and Svennerholm (1971)

Table 19 (Continued)

Fatty acids	Results	References
$C_{18:1}$, $C_{20:1}$ aldehydes	decreased	Winterfeld and Debuch (1977); Arnetoli et al., (1969)
mono-enoic acid in SM	normal	Clausen and Hansen (1970)
$C_{24:1}$ in cerebroside	increased	Alling et al. (1971)
	decreased	Gerstl et al. (1970)
Polyenoic acid in PE, PC and other glycero-phosphatides	normal	Fewster and Hirono (1976)
$C_{18:3}$, $C_{22:3}$, $C_{24:1}$ in total lipid fraction	decreased	Boggs and Mascarello (1980)
FA content	increased	Clausen and Hansen (1970)
$C_{22:4}$ of PE	decreased	Geopfert (1980)
$C_{20:1}$ of PS	decreased	Geopfert (1980)

lipid change in demyelination is the formation of cholesterol esters, though the significance of this finding in the mechanism of demyelination is still unclear.

The unsaturation of C_{18} fatty acids was reported to be decreased (Amaducci, Pazzagli and Petruzzi, 1965).

In a recent study of the sphingolipid of the MS plaques (Yahara, 1982), the non-hydroxycerebroside, hydroxycerebroside, non-hydroxysulfatide and hydroxysulfatide were decreased by over 99%, but the relationship with the demyelination process was not discussed.

Although there are many conflicting reports about the fatty acid profile in normal looking white matter and plaques a general trend can still be established. Certain mono- or polyunsaturated fatty acids were low in one or other of the fractions isolated from MS brains. This supports the hypothesis that the myelin sheath may be chemically altered during development, principally due to a relative dietary deficiency of unsaturated acids. Others suggest that the fatty acid changes observed in the diseased brain reflect some difference in lipid metabolism leading to altered myelin either during initial synthesis or normal maintenance (Smith and Thompson, 1977).

1.5.1.5 CSF

It has been suggested that lipid changes in CSF may originate from lymphocytes and serum due to dysfunction of the blood-brain barrier. So any changes rather reflect the changes in serum or blood cells and indicate a dysfunction of blood-brain barrier, than reflect a local change in CSF.

Increase of SM in CSF was thought to originate

from disintegrated lymphocytes, (Seidel, 1980). Linoleate was found to be increased in CSF, which was suggested to come from serum due to dysfunction of the brain-blood barrier, although the same acid was found to be decreased in serum (Seidel, 1980; 1981). Pedersen (1974) reported a decrease in phospholipid and PE, but an increase in esterified cholesterol in CSF of MS patients

1.5.2 Enzymes

1.5.2.1 Red cells, lymphocytes and serum

Decreased erythrocyte glutathione peroxidase (GSH-Px) activity has been reported by Szeinberg (1979) who studied Israeli Jews and Shukla, Jemsen and Clausen (1977) who examined a Danish group. Both groups suggested a selenium deficiency might be the cause of the observed decreased GSH-Px activity since selenium is an essential part of the enzyme. However, Jensen (1980) found increased selenium in MS red cells but normal levels in serum or whole blood. Another enzyme which is involved in protection against peroxidation, glutathione reductase (GR) was reported to be slightly decreased (but non-significantly) in red cells (Jensen, 1980). These findings suggest that susceptibility to membrane lipid peroxidation is increased. If these changes in red cells reflect the same changes in CNS, these findings may have important significance in the pathogenesis of the disease. It has been shown that in rats, GSH-Px activity in red cells increases in parallel with that of brain during development (Sinet, Dejeune and Jerome, 1979).

In serum, increased β-D-glucuronidase activity and

abnormal γ-glutamyl transferase were reported (Jensen, 1980; Rieder, Zurfluh and Jegge, 1977). The latter change might reflect disturbed liver function, but no liver malfunction has even been associated with MS.

Demyelination has been considered to be the result of increased hydrolytic enzyme activities. The source of those enzymes was suggested, in part, to be lymphocytes which infiltrate from a vein into the lesion region. Some lysosomal enzymes, e.g. acid phosphatase were increased in granulocytes and β-glucuronidase was also increased in lymphocytes during the relapse period (Riekkinen, and Palo, 1977). They suggested lysosomal enzymes were activated by some unknown mechanism during demyelination. Cuzner, Davison and Rudge (1978) demonstrated increased neutral proteinase but decreased acid proteinase in MS leukocytes. Rastogi and Clausen (1981) studied the soluble enzymes of peripheral polymorphonuclear leukocytes and found they degraded more basic protein and Wolfgram protein in MS myelin than normal myelin.

1.5.2.2 CNS

It is generally accepted that the observed increased acid proteinase activity in and around the plaques is responsible for the degradation of myelin basic protein (Einstein, Dalal and Csejtey, 1970; 1972; Riekkinen, Clausen, Frey, Fog and Rinne, 1970; Cuzner and Davison, 1973). Einstein et al. (1972) observed a gradient of acid proteinase activity, increased from control white matter with distance from the centre of the plaque. They proposed the enzyme might be derived from lysosomes in the

neuroglial cells which proliferate at the rim of active plaques. The smaller encephalitogenic peptides that are released from the degraded myelin basic protein would be able to leave the brain and stimulate immunoreceptor sites and the resulting antibody or sensitized cells (or both) could provoke further vascular and myelin damage (Einstein et al., 1972).

Other changes in the lysosomal and other enzymes in the plaques are summarized in the following table (Table 20).

The increase in phosphatase and phospholipase A_2 may explain phospholipid breakdown in myelin while decreased activity of cholesterol ester hydrolases was considered to be the cause of the generalized increase of cholesterol ester in the plaques and myelin. However, the mechanism of the activation of these lysosomal enzymes and their source are still obscure and is an important question to be solved for the understanding of the actual mechanism of demyelination.

1.5.2.3 CSF

The source of the enzymes in CSF in MS patients is probably largely from regions of active demyelination.

Neutral and acid proteinase have been found to be raised in CSF (Cuzner, Davison and Rudge, 1978; Richardo, Cuzner, and Cuzner, 1978). 2'3'-cyclic nucleotide 3'-phosphohydrolase is absent in normal CSF but was found to be increased in CSF from MS patients (Banik, Mauldin and Hogan, 1979; Eickhoff and Heipertz, 1979). Johnson and Shah (1981) observed decreased activity of neutral cholesterol ester hydrolases in CSF from patients with MS and this enzyme may be responsible for the increased cholesterol ester observed in CSF and in CNS.

Table 20 Enzymes in the Plaques of MS Patients

Enzymes	Observation	Proposed source	References
β-glucuronidase	increased		Cuzner and Davison, 1973
arylsulphatase	increased		
acid phosphatase	increased	astrocytes or macrophages	Hirsch, Duquette and Parks, 1976; Allen, 1979
acid lipase-esterase	decreased		
lactic dehydrogenase	increased		
ester hydrolyses	decreased		Shah and Johnson, 1980
esterifying enzyme	decreased		
phospholipase A_2	increased		Woelk, Peiler-Ichikewa, 1974
cholesterol esterifying enzyme	decreased		Shah, Johnson, 1980
cholesterol ester hydrolase	decreased		
Cyclic AMPase	decreased		Geopfert, Pytlik and Debuch, 1982

1.5.3 Basic protein and its fragments in CSF and plaques

Basic protein is the main protein in myelin and during demyelination, breakdown fragments of basic protein are released into CSF, and act as an indicator of myelin breakdown (Gutstein and Cohen, 1978; Cohen, 1978; Bashir and Whitaker, 1980). Carson, Barbarese, Braun and McPherson (1978) detected these components in CSF by radioimmunoassay for myelin basic protein. In addition to intact myelin and proteolytic fragments of myelin basic protein, a protein of unknown origin was found, which was specific to MS patients. They proposed this unknown protein might be related to demyelination and might be one of the following, (i) an abnormal form of myelin basic protein in myelin which caused it to break down, (ii) a modified form of myelin basic protein produced during demyelination or (iii) a nonmyelin protein which, because of its antigenic similarity to myelin basic protein, induced an autoimmune response directed against the patients' myelin. But the antigenic nature of this unknown protein and its relationship with demyelination has not been confirmed.

Polyacrylamide gel electrophoresis of plaque proteins shows extensive loss of myelin proteins (especially basic protein) and an equally significant increase in a high molecular weight peptide fragment which might be the breakdown products of basic protein (Newcombe, 1980). As in CSF, this is an indicator of active demyelination.

1.5.4 Serum factors

A serum factor from MS patients was found to inhibit RNA synthesis of normal lymphocytes (Knowles, Hughes,

Caspary and Field, 1968; van den Noort and Stjernholm, 1971) but this effect could not be reproduced by others (Anderson, Sweet and Armentrout, 1979). This factor has been identified as a lymphotoxic factor. A more accurate and specific designation is lymphocyte transformation inhibition factor (LTIF). LTIF inhibited the blast transformation of normal cultured lymphocytes stimulated by phytohaemagglutinin (PHA) (Cendrowski and Niedziekka, 1970; Anderson et al., 1979) or in the presence (Armentrout, Sweet and Galant, 1981; Jenen, 1968) or absence (Schocket, Weiner, Walker, McIntosh and Kohler, 1977) of other mitogens. Anderson et al. (1979) indicated that LTIF inhibited RNA protein and DNA synthesis, in later stages of the transformation. This factor has been isolated and identified by White, Armentrout and van den Noort (1975). It is a protein with small molecular weight, heat stable, nondialyzable and was transient, being most often found during exacerbation and with high grades of disability. T-cells were particularly sensitive to this factor (White et al., 1975; Armentrout et al., 1981). LTIF may be a humoral component found in MS patients, as an important modulator of the immune system (Armentrout et al., 1981). In addition a serum factor is thought to be responsible for the abnormal electrophoretic mobility seen in erythrocytes incubated with linoleate (see section 1.3.3.1).

1.5.5 Immunoglobulins in cerebrospinal fluid (CSF)

In the majority of MS patients, the level of IgG in CSF is elevated (Kabat, Glusman and Knaub, 1948). In MS, IgG is synthesized locally, i.e. within the CNS.

The CSF IgG in MS is relatively homogeneous in charge and following electrophoresis is distributed in a small number of discrete (oligoclonal) bands (Johnson and Nelson, 1977). The oligoclonal pattern shows the highest incidence of any abnormal finding in MS (Thompson, 1977).

It is a consistent finding that in about half the MS patients the K:λ ratio for light chains in serum is normal (i.e. 1.0), but the ratio is higher (approximately 2.0) in CSF (Thompson, 1977). When the B lymphocytes from CSF were typed, they showed the same elevated K:λ ratio for light chains (Sandberg-Wollheim and Turesson, 1975).

1.6 Physical Properties

1.6.1 Red cells

An increase in red cell fragility in MS patients has been reported by several laboratories (Stasiw, Rosato, Mazza and Cerry, 1977; Schauf, Frischer and Davis, 1980; Kurantsin-Mills, Sainji, Moscarello and Boggs, 1982). Caspary, Sewell and Field.(1967) reported active cases of MS had more fragile red cells than the inactive ones which were indistinguishable from controls.

A recent study (Pollock, Harrison and O'Connell, 1982) on red cell deformability demonstrated no significant difference between MS patients and normal. An increase in diameter in red cells from patients with MS was reported by Prineas (1968). Kurantsin-Mills et al. (1982) reported normal membrane fluidity and morphology in red cells from MS patients.

The changes in the physical properties in red cells tell little about the pathogenesis of demyelination, but may be related to the increased platelet stickiness reported since platelet stickiness is known to be influenced by the adenosine diphosphate (ADP) liberated by the red cells.

1.6.2 Platelets and myelin

Caspary, Prineas, Miller and Field (1965); Sanders, Thompson, Wright and Zilkha (1968); Wright, Thompson, and Zilkha (1965) reported increased platelet stickiness in MS patients and Sanders et al. (1968) found a significant inverse correlation with cholesteryl linoleate in serum, which

is absent in healthy controls. Similarly, Wright et al. (1965) proposed that the increased platelet adhesiveness might be due to lowered linoleate level in the serum. Field and Caspary (1964) studied platelet stickiness by a different approach. They found addition of a highly encephalitogenic factor derived from human brain to plasma of MS patients in an acute phase increased the stickiness of their platelets and the stickiness ran parallel with the activity of the disease process. Surprisingly, addition of encephalitogenic factor to normal human serum decreased platelet adhesiveness.

Besides proposing that lowered linoleate in serum explains the increased platelet stickiness observed in MS patients, Prineas (1968) and Caspary et al. (1967) related increased platelet stickiness to changes in size and fragility in MS red cells. They suggested the changes in these physical properties might imply some change in RBC membrane properties, probably involving phospholipid, which would in turn allow liberation of excess ADP or other factors from red cells, resulting in increased platelet stickiness. Caspary et al. (1967) discussed the important significance of increased platelet stickiness in the pathogenesis of MS. They indicated that platelet aggregation might occlude the small veins within the nervous system and lead to reversible or irreversible changes, depending on degree and duration of obstruction. This theory is reminiscent of that of James (1982) and provides a more plausible cause of blood vessel occlusion. However, the criticism that no abnormal vascular pathology has been observed is equally applicable

to this idea.

Boggs and Mascarello (1980) studied myelin fluidity by ESR and fluorescence polarization spectroscopy and found there was no difference in fluidity between MS myelin from normal-looking white matter and normal myelin. This might imply there is no gross change in the physical state of the lipids of MS myelin before demyelination.

1.7 Immunological Findings

1.7.1 Viruses

The idea that MS could be a slow virus disease or more likely, a virus-induced immune disease came from the finding that there is the presence of a measles virus-specific IgM in the serum and the oligoclonal synthesis of IgG in brain, part of which is measles virus specific (Fraser, 1977) in MS patients.

Attempts to isolate the virus have met with little success.

The results are summarized in the following table (Table 21).

Prineas (1972) suggested that MS lesions might be initiated by seeding of lymphocytes bearing latent paramyxovirus to white matter of the CNS. Viruses can modify major serologically determined histocompatibility antigens (Doherty and Zwikernagel, 1975), which will become targets for cytotoxic reactivity (Schrader, Cunningham and Edelman, 1975). Thus the interaction of a virus with neural tissue in MS could alter antigenicity, causing the induction of an immune reaction. If a virus is involved in the pathogenesis of MS, it may well be a latent persistent virus, since the intermittent advance shown by MS may be related to activation and spread of a persistent virus (Fraser, 1977). The presence of a virus in MS patients is further suggested by the changes in cellular immunity and humoral factors observed in MS patients.

Table 21 Viruses Isolated in CNS of MS Patients

Virus	Source	Reference
virus 6/94 (type 1 parainfluenza virus)	MS brain cultured cells	Lewandowski, Waters and Koprowski, 1973
gp-1 parainfluenza virus	fusion of MS brain tissue culture cell with indicator cell	Menlen, Koprowski, Iwasaki, Kächell and Müller, 1972
paramyxovirus nucleocapsids	mononuclear cells infiltrating the zone of lesion	Prineas, 1972
a thermolabile filterable virus	CSF	Melnick and Seidel 1982

The results are inconsistent and highly doubtful as only one or two MS patients were used.

1.7.2 Cellular immunity

There are three properties of the immune system in MS that have led to the theory that cell-mediated immune mechanisms may be involved in the production of the disease:

(1) Lymphocytes are present at the sites of the lesions.

(2) There are changes in the types of lymphocytes and their functions both in vitro and vivo during the course of the disease.

(3) There are similarities between MS and some known autoimmune diseasesboth in human beings and animals.

Some abnormalities were found in the lymphocytes, which might be virus-induced or genetically determined. A normal distribution of B and T cells in MS patients was found by Lamoureux, Giard, Jolicoeur, Toughlian and Desrosiens (1976) and Santoli, Moretta, Gilden, Lisak, Koprowski (1978) and Nowak and Wajgt (1975) but Lisak, Levinson, Zweiman and Abdon (1975) reported an increase in B cells but decreased T cells. In a study of the subpopulation of T cells, Santoli et al. (1978) indicated the T.G cells (T cells which bind IgG immune complexes) were increased while Nowak and Wajgt (1975) found a significant reduction in lymphocytes bearing C_3 receptor in MS patients. These abnormalities might be genetically determined defects. A suppressed cellular response to viruses in MS have also been reported. Both Utermohlen and Zabriskie (1973) and Neighbour and Bloom (1979) found a suppressed cellular response and decreased amount of interferon produced by MS lymphocytes in response to measles virus.

The cause of this may be an initial measles infection which results in the elimination of a clone of cells reacting to measles and thus in suppression of cellular reactivity to the antigen. This may permit persistence of measles in CNS and on going lateral infection (perhaps directly in astrocytes) and may result in demyelination. An alternative explanation of the suppressed cellular immune responses observed in MS patients might be that they are genetically determined. MS patients were also unable to make an effective antibody against measles. As measles persisted the patient was stimulated to make a higher titre of ineffective antibody, continuously. These high measles antibody titres might then suppress cellular reactivity to this antigen (Utermohlen and Zabriskie, 1973).

Macrophages, another cell type which is involved in the production of immune responses, were found to be increased in number in the nervous system in MS patients (Bowen and Davison, 1974), these cells could be important as a reservoir of virus or in transporting the virus to target sites in the body of MS patients. (Waters, Koprowski and Lewandowski, 1975).

The abnormalities observed in the cellular immune response and its components, either due to a genetic error or virus induced, have a close relationship with measles virus. These phenomena emphasize the importance of measles virus in the pathogenesis of MS and make this an interesting area to be explored.

1.7.3 Humoral factors

Immune-mediated inflammation can lead to demyelination in MS if directed against myelin or a myelin component, or even, more speculatively against a virus antigen associated with myelin or oligodendroglial cells thus causing the characteristic plaques seen in MS (Caspary, 1977). Increased antibody (Ab) against various viruses was found in the body fluids of MS patients. Greater than 90% of MS patients show oligoclonal γ-globulin bands in CSF (Thompson, 1979), part of which is measles specific (Fraser, 1977). Locally synthesized Abs against measles, rubella, mumps and herpes simplex virus were observed in MS CSF (Nordal, Vendvik and Norrby, 1978) and brain (measles only) (Rastogi, 1979). Similar Abs were also found in serum of MS patients. In fact, the frequency of measles Ab was significantly greater in MS group than in the control group (Brown, Cathola, Gajdusck and Gibbs, 1977). Other increased Ab against viruses in MS patient serum were against rubella (Synington, Mackay, Wittingham, White and Backley, 1978), vaccinia (Brody, Sever, and Henson, 1971) varicella (Lamoureux, Giard, Jolicoeur, Tonghlian and Desrosiers, 1976) and EB-virus (Sumaya, Myer and Ellison, 1980).

The only Ab which is most consistently found in MS patients is against measles, implicating the close relationship of this virus with the pathogenesis of MS.

Ab against nervous system components was also reported. Michetti, Massaro and Murazio (1979) observed a nervous system specific S-100 Ab in CSF in MS which was

absent in the control group. The amount of this Ab seemed to correlate with the acute state of the disease. After elution of MS brain a higher content of immunoglobulin (Ig) was found in the eluate (Gilden and Tachovsky, 1979). During relapse, there was increased intracerebral IgG synthesis in MS patients (Ewan, Lachmann, 1979). In serum of MS patients, there was an antimyelin Ab (Lisak, Sweimar and Norman, 1975). On the other hand, a myelinotoxic factor was found in MS patients serum, and its activity seemed parallel to the severity of the disease (Caspary, 1977). A recent study (Lebar, Boutry, Vincent, Voisin and Robineaux, 1976) showed that the demyelinating effect of this myelinotoxic factor was due to complement -fixing auto-antibody of the IgG_2 class. This Ab was not directed against myelin basic protein, but apparently against cerebroside.

To summarize the disease is probably initiated by a virus (probably measles) infection in early life and much of the destruction in the nervous system involves an inflammatory immune reaction. The possible existence of a genetic error in immune system may control susceptibility and response to the initial infection and also the immune response to antigen potentially capable of causing inflammatory demyelination. An autoimmune response against a virus persistent in the CNS, or more precisely, the virus-induced change in surface antigens of the CNS might be responsible for the demyelination as suggested by the presence of anti-virus Ab and anti-nervous system Ab in serum and CNS of the MS patients. However, evidence for an

aggressive immune demyelinating process in MS is far from complete.

1.8 Membrane Lipid Peroxidation

1.8.1 General features

Peroxidation of membrane lipids is an autocatalytic, free radical - mediated series of reactions. It occurs both in normal physiological processes e.g. prostaglandin synthesis, phagocytosis and aging, and in abnormal conditionse.g. haemolytic anaemia, lung damage, liver necrosis and muscle atrophy (McCay, 1981).

Polyunsaturated fatty acids (PUFA) occur most frequently in membranes. PUFA have a high potential to form lipid peroxides because the double bonds are particularly susceptible to hydrogen abstraction, resulting in the formation of a fairly stable free radical and, in the presence of O_2, in the initiation of a radical chain leading to autoxidation (Mead, 1980).

Under normal conditions, the extent of membrane lipid peroxidation is very small and non-damaging due to (i) the operation of efficient protective mechanisms; (ii) the normal segregation in the cell of the principle reactants of the radical chain process; and (iii) the normally low partial pressure of oxygen in most tissues. All of these factors serve to limit the existence of peroxides to a very transitory one and to prevent the efficient propagation of radical chains (Mead, 1980). However, under pathological conditions where there is either an overproduction of oxidants and/or free-radical generating species or a deficiency in one or more protective mechanisms, extensive membrane damage can occur.

1.8.2 Cellular damage due to lipid peroxidation and free radicals

Lipid peroxidation in membranes can bring about lipid-lipid or lipid-protein crosslinking; protein damage (e.g. enzyme inactivation) and loss of membrane integrity (Mead, 1980).

Free radicals, especially hydroperoxide radicals (ROO·) occur during lipid peroxidation and can cause haemoglobin to breakdown by oxidizing thiol (-SH) groups. (Aebi and Suter, 1974). One of the break-down products can bind to the red cell membrane resulting in increased osmotic fragility (Goldbery and Stern, 1977).

Essential thiol groups of enzymes can be oxidised which will cause a change in conformation and thus inhibition of the enzymes function (Slater, 1972).

Membrane lipid peroxidation causes a loss of specialised membrane function (e.g. in mitochondria) and will cause production of malondialdehyde, a breakdown product of lipid peroxidation, which can cross-link proteins, lipids and DNA causing chromosomal abnormalities, membrane damage as well as inhibiting enzymes by altering their structure (Slater, 1972). Thus, uncontrolled lipid peroxidation is extremely damaging.

1.8.3 Potential intracellular oxidants

The potential intracellular oxidants mostly are the derivatives of activated oxygen. They are interconvertible and are continually formed in the cell. The main oxidants are superoxide ($O_2^{\cdot -}$), peroxide (O_2^{2-}), singlet oxygen ($^1O_2$)

and most reactive of all is the hydroxyl radical ($OH^{\cdot -}$) (Halliwell, 1978).

Halliwell (1978) suggested that activated oxygen was produced in the four step reduction of oxygen to water.

$$O_2 \xrightarrow[\text{energy}]{} O_2^{\cdot -} \xrightarrow[E]{} O_2^{2-} \xrightarrow[E]{} OH^{\cdot -} \xrightarrow[E]{} H_2O$$

Superoxide is easily convertible to other activated species:-

$$O_2^{\cdot -} + O_2^{\cdot -} \xrightarrow{\text{dismutation}} {}^2O_2^{=} + {}^1O_2$$

$$O_2^{\cdot -} + H_2O_2 \longrightarrow \cdot OH + OH^- + O_2$$

Other sources of these oxidants in the red cell are (Halliwell, 1978):-

(1) Autoxidation of oxyhaemoglobin to methaemoglobin can cause displacement of superoxide $O_2^{\cdot -}$ if a random fluctuation in the haem pocket of haemoglobin allows water to enter. Any factor which increases flexibility or distortion of the haem pocket will increase $O_2^{\cdot -}$ production.

(2) Heavy metal ions (especially those with a divalent charge) will react with oxyhaemoglobin. One of the products is $O_2^{\cdot -}$.

(3) Free porphyrins can ultilise ultraviolet radiation to activate oxygen, probably to singlet oxygen ($^1O_2$).

(4) Oxidases e.g. Xanthine oxidase produce $O_2^{\cdot -}$.

1.8.4 Chemistry of PUFA peroxidation

PUFA can undergo a hydrogen abstraction to give a radical which will initiate lipid peroxidation in the presence of oxygen (Witting, 1980).

(i) Initiation

$$(\text{-CH=CH-CH}_2\text{-}) \xrightarrow{-H} (\text{-CH=CH-}\dot{\text{C}}\text{H-})$$

PUFA — free radical initiator (R·)

(ii) Propagation

$$(\text{-CH=CH-}\dot{\text{C}}\text{H-}) + O_2 \longrightarrow (\text{-CH=CH-CH(O-}\dot{\text{O}}\text{)-}) \quad (RO_2^{\cdot})$$

FA peroxyradical

$$(\text{-CH=CH-CH(O-}\dot{\text{O}}\text{)-}) + (\text{-CH=CH-CH}_2) \longrightarrow (\text{-CH=CH-CH(O-OH)-}) + (\text{-CH=CH-}\dot{\text{C}}\text{H-})$$

PUFA — hydroperoxide

Chain reaction

$$(\text{-CH=CH-CH(O-OH)-}) \xrightarrow[\text{oxidation}]{\text{secondary}} CH_2(CHO_2) \text{ etc.} \longrightarrow \text{tissue damage}$$

malondialdehyde

(iii) Branching

$$2(\text{-CH=CH-CH(O-OH)-}) \xrightarrow{OH^-} (\text{-CH=CH-CH(O-}\dot{\text{O}}\text{)-}) + (\text{-CH=CH-CH(O}\cdot\text{)-})$$

$[R\cdot]$ $[R\cdot]$

free radical multiplication

(iv) Termination

$$2\ R\cdot \longrightarrow R\text{-}R$$

$$2\ RO_2^{\cdot} \longrightarrow O_2 + ROOR$$

$$RO_2^{\cdot} + R^{\cdot} \longrightarrow ROOR$$

} polymeric peroxides

(Witting, 1980)

Antioxidants, e.g. vitamin E can break the propagation of the chain reaction mainly by scavenging the free-radicals (Horwitt, 1965) and terminating the chain reaction as above.

1.8.5 Protective mechanisms

Cells contain several protective mechanisms against lipid peroxidation. These consist of several enzymes and antioxidants which act at different stages.

1. Superoxide dismutase (SOD)

There are three types of SOD, classified by the metal ions associated with them: Cu/Zn SOD (found in eukaryote cytosol), Mn-SOD (in eukaryotic mitochondria and prokaryotes) and FeSOD (prokaryotes) (Bannister and Bannister, 1981).

SOD catalyzes the following reaction:

$$2\ O_2^{\cdot -} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

superoxide radicals

(Witting, 1980)

In red blood cells SOD and catalase are collected in the same fraction as haemoglobin during repeated washings of lysed cells, indicating they are both in the cytosol. It is suggested that one important function of SOD and catalase is to act against bursts of oxygen radicals formed from oxyhaemoglobin (Cohen and Hochstein, 1963).

SOD is a very stable enzyme and is resistant to proteolysis and inactivation by heat (Fridovich, 1974).

2. Catalase

Catalase destroys the hydrogen peroxide (H_2O_2) produced by the action of SOD and other reactions

$$2\ H_2O_2 \xrightarrow{\text{catalase}} 2\ H_2O + O_2$$ (Tappel, 1978)

As indicated above the main purpose of red cell catalase is to decompose H_2O_2 formed as a result of bursts of oxygen radicals formed by oxyhaemoglobin.

Interaction of oxyhaemoglobin with metabolites or drugs can cause bursts of $O_2^{\cdot-}$ followed by H_2O_2. The K_m of catalase for H_2O_2 is considerably higher than that of glutathione peroxidase. Therefore, catalase of red cells would be better for scavenging sudden elevated H_2O_2 rather than low steady state levels of H_2O_2 (Cohen and Hochstein, 1963).

3. Glutathione Peroxidase (GSH·Px)

GSH·Px is a tetramer of MW≈84,000. It has no haem or flavin prosthetic groups which is unusual for peroxidases. Each subunit has a selenium atom associated with it which is essential for catalysis (Flohe and Gunzler, 1974).

The reaction catalyzed is:

$$2\ GSH + H_2O_2\ (\text{or } ROOH) \longrightarrow GSSG + H_2O\ (\text{or } ROH)$$

reduced glutathione — peroxyacid — oxidised glutathione — hydroxyacid

(Witting, 1980).

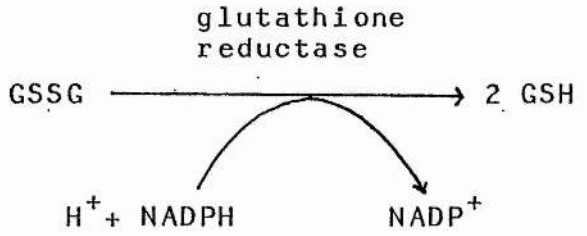
It scavenges H_2O_2 and destroys lipoperoxides preventing ·OH formation (Westermarck, 1982).

This enzyme is highly specific for GSH as the hydrogen donor (Flohe and Gunzler, 1974).

4. Glutathione reductase (GR)

This enzyme is important for recycling reduced glutathione (GSH) which is converted to oxidized glutathione (GSSG) by GSH·Px.

The reaction is:

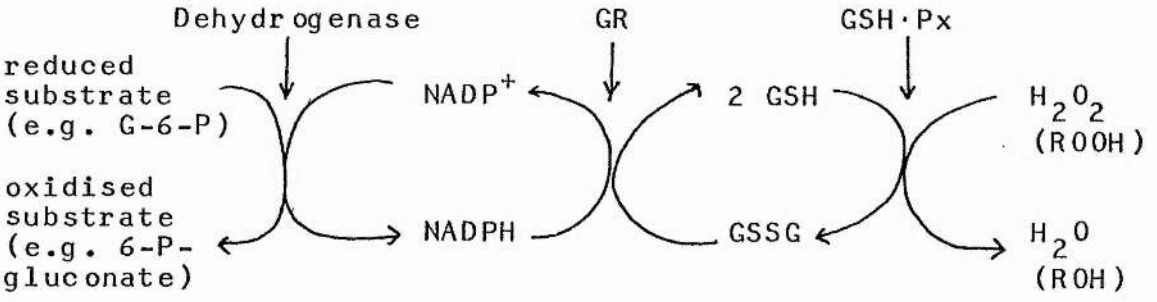


The enzyme is not specific for acceptor substrates and will reduce other disulphides as well as GSSG, although at a much lower rate (Beutler, 1974).

5. NADPH generating dehydrogenases

As can be seen above NADPH is essential for glutathione reductase to generate GSH which is in turn an essential substrate for GSH·Px. Most of the NADPH is generated by the dehydrogenase enzymes of the pentose phosphate pathway i.e. glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (Aebi and Suter, 1974).

The reactions which link GSH·Px, GR and dehydrogenases are shown below:



(Cohen and Hochstein, 1963)

6. Antioxidants e.g. Vitamin E (α-tocopherol)
Vitamin C (ascorbic acid)

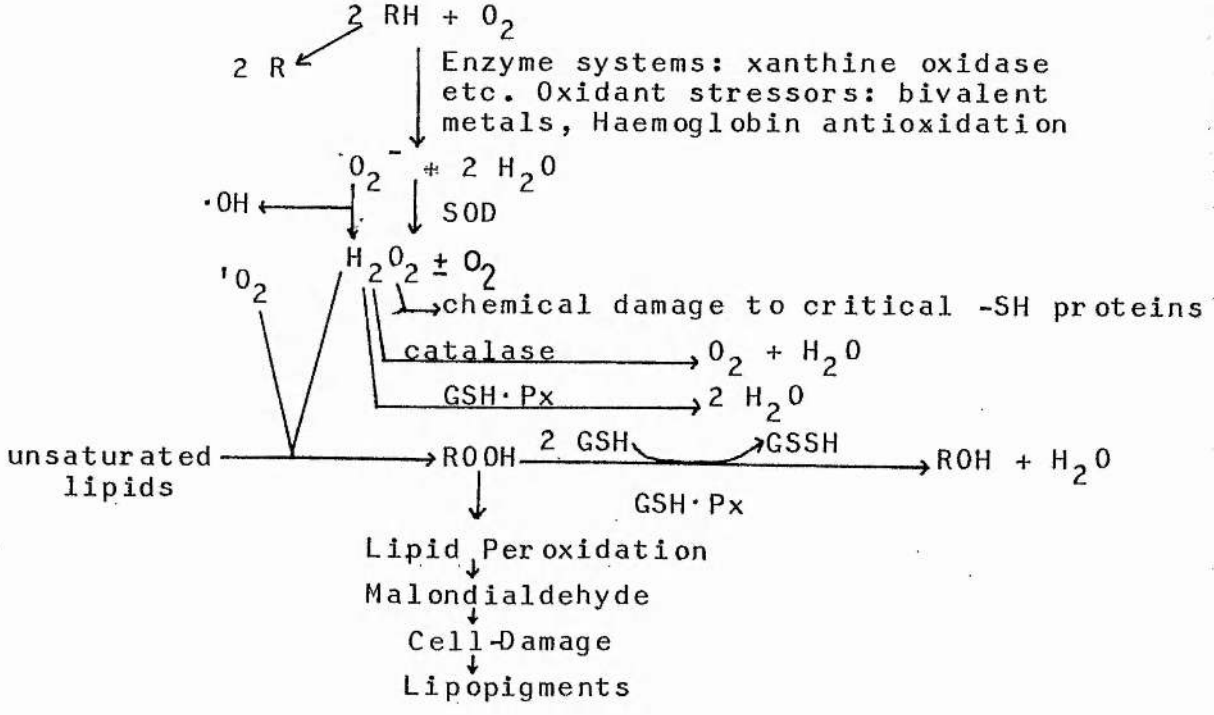
The primary physiological function of vitamin E is not clearly understood yet. It is thought that vitamin E

has an important role in terminating peroxidative reactions of PUFA, because of its free radical scavenger ($R\cdot \longrightarrow RH$) character and lipophilic structure which enable vitamin E to participate in a strong physical interaction with PUFA in the membrane (Konings and Drijver, 1979). It will also reduce H_2O_2 becoming a more stable and less reactive tocopherol radical (Halliwell, 1978). Vitamin C is a similar antioxidant acting as a free radical scavenger (Bannister and Bannister, 1981) in the water-soluble phase.

Selenium also appears to act as an antioxidant in its own right but is important in the reaction of GSH·Px (Metzler, 1977).

There are many other substances in cells which also act as antioxidants to protect the cell from oxygen stress and lipid peroxidation.

Summary of potential oxidative mechanisms and enzymic protection against peroxidation (After Westermarck, 1982):



1.9 Possible Links Between MS and Membrane Lipid Peroxidation

Shukla et al. (1977) and Szeinberg (1979) found that GSH·Px was significantly decreased in MS red cells and suggested that the susceptibility to lipid peroxidation might be increased in MS patients, which may be the cause of the pathological changes in MS.

Finland has a high incidence of MS which is associated with low levels of selenium (Alter and Olivares, 1970).

A higher incidence of MS is seen in populations who consume a high proportion of animal fat in their diet which would be associated with a reduced vitamin E intake since vitamin E is found predominantly in association with plant lipids (Belin, 1971).

CNS myelin of MS patients has decreased concentrations of the acidic phospholipid classes (see 1.5.1.4). This could be due to their preferential peroxidation as these classes contain the highest proportion of PUFA (Clausen, 1970).

Mickel (1975) suggested MS may be attributable to lipid peroxide absorption from the gut. These could cause fragility of lysosomal membranes, release of lysosomal peroxidases and enhance autoxidation of myelin PUFA. Oligodendroglia may have little antioxidant and thus be especially susceptible to the action of lipid peroxides.

1.10 Aims of This Work

Although the lipid composition of MS red cells was found to be normal (Homa et al., 1980), it is still possible that the lipid organizations within the two halves of the bilayer is perturbed, and that this might lead to the observed change in physical proporties (see 1.6.1) in MS red cells. It is also possible that the transbilayer distribution of phospholipids in MS red cells may give some information about lipid organization in myelin. The organisation of erythrocyte membrane phospholipids was examined using bee venom phospholipase A_2 as in Chapter I.

In the light of the evidence given above, it would be reasonable to suggest that membrane lipid peroxidation is involved in the pathogenesis of MS. Membrane lipid peroxidation could account for many of the changes seen in MS especially those in the red cells.

In order to test this hypothesis protective mechanisms against membrane lipid peroxidation were examined to see whether any deficiency might result in increases susceptibility to peroxidation damage. If protective enzymes were found to be increased, this might also represent an induced response to increased membrane lipid peroxidation. The H_2O_2 stress test, a direct measurement of lipid 'peroxidisability' (i.e. antioxidant capacity) in red cells, and assays of the four protective enzymes, i.e. - SOD catalase, GR and GSH Px were carried out in this work.

James (1982) proposed that the cause of the

demyelination in MS may be fat emobi (for details see 1.4.1). Hyperbaric oxygen (HBO) treatment has been the subject of controlled clinical trials and is currently being used in several countries. The effect of HBO treatment on the four antioxidant protective enzymes was also investigated.

2. MATERIALS AND METHODS

2.1 The Patients

All patients had definite MS according to the McAlpine criteria; they are or have been patients at Dundee Royal Infirmary. Controls were healthy volunteers with no family history of MS. Patients' (9F, 5M) ages ranged from 25-61 whilst those of the controls (5F, 13M) were 20-42.

2.2 Asymmetric Lipid Organization of RBC Membrane

The procedures were exactly as described in Chapter I, sections 2.2. - 2.9.

2.3 Lipid Peroxidation in MS RBC

2.3.1 Isolation of washed packed RBC

This was carried out exactly as in section 2.2. of Chapter I.

2.3.2 Haemoglobin Determination

The method of Dacie and Lewis (1975) was followed. The following were made up to 1 L with distilled water: 200 mg potassium ferricyanide; 50 mg potassium cyanide; 140 mg KH_2PO_4; 1 ml nonidet P_{40}. 0.1 ml packed cell suspension was diluted 200 x with the above reagent and the $O.D._{540}$ read against a reagent blank. The haemoglobin concentration can be calculated from the following expression:

$$\text{Hb (mg/L)} = \frac{O.D._{540} \times \text{M.W. Hb} \times \text{dilution factor}}{\text{molar extinction coefficient} \times d}$$

molecular weight of Hb = 64500

dilution factor = 200

molar extinction coëfficient = 44.0

path length (d) = 1

2.3.3 Hydrogen Peroxide Stress Test (Stocks and Dormandy, 1971)

2.3.3.1 Principle

An accelerated form of non-enzymic oxidative breakdown was induced in red cells by exposure to hydrogen peroxide (H_2O_2). The formation of malonyldialdehyde (MDA), a secondary fragmentation product of polyunsaturated fatty acid peroxides, can be estimated as a measure of polyunsaturated fatty acid (PUFA) autoxidation in red cells

incubated with H_2O_2 in vitro. This is therefore an indirect way to measure lipid autoxidation by assaying the colour developed from MDA-TBA (thiobarbituric acid) colour reaction.

Reactions:

$$O_2^{\cdot -} + H_2O_2 \longrightarrow \cdot OH + OH^- + O_2$$

($O_2^{\cdot -}$: (superoxide radical generated from the conversion of oxyhaemoglobin to methaemoglobin)

$$RH + \cdot OH \longrightarrow R\cdot + H_2O$$

(RH: PUFA)

$$R\cdot + O_2 \longrightarrow RO_2^{\cdot}$$

$$RO_2^{\cdot} + RH \longrightarrow ROOH + R^{\cdot}$$

$$ROOH \longrightarrow CH_2(CHO_2)$$

(2^0 oxidation; $CH_2(CHO_2)$: MDA)

$$MDA + TBA \longrightarrow \text{red-orange colour complex}$$

(condensation)

Sodium azide was added to inhibit catalase activity, which otherwise will breakdown the H_2O_2 added very rapidly, also help to preserve the oxidation product (MDA). Under these conditions, the peroxide stress test measures the antioxidant capacity of the red cells. Since catalase is inhibited and the relatively high H_2O_2 concentration is vastly in excess of the K_m for glutathione peroxidase, it is likely that the antioxidants involved would be at a later stage in the overall process than H_2O_2 destruction. In fact, this test correlates well with the vitamin E status of some, though not all, individuals. Trichloroacetic acid (TCA) was added to precipitate the cells, which were removed before boiling, preventing the coloured complex

formed between haem compounds and TBA, and between β-formylpyurvic acid and TBA (absorption maximum 550 nm) in the TCA boiling mixture. The addition of arsenite at the end of the incubation stage stabilizes the MDA chromogen.

2.3.3.2 Reagents

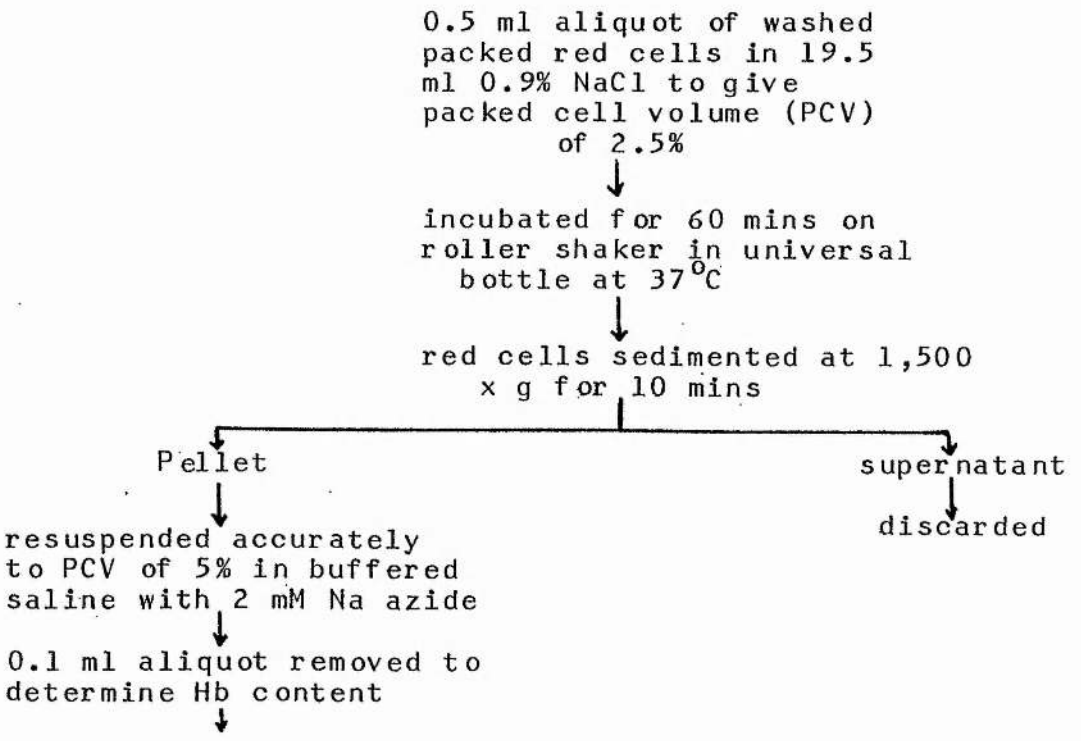
1. 0.9% NaCl
2. Reagent I

   1 volume of 0.15 M KH_2PO_4/0.15 M K_2HPO_4 (pH 7.4) mixed with 9 volumes of 0.9% NaCl.
3. 2 mM sodium azide in Reagent I.
4. 20 mM H_2O_2 in Reagent I.
5. Reagent II

   28% (w/v) trichloroacetic acid (TCA) and 0.1 M sodium arsenite.
6. Reagent III

   1% (w/v) thiobarbituric acid (TBA) in 0.05 M NaOH.

2.3.3.3 Procedure



↓

equal volume of 20 mM H_2O_2 in buffered saline added

↓

incubated at 37°C on a roller shaker

↓

4.0 mlsuspension withdrawn at 10, 20, 30, 60, 90, 120 mins. intervals for MDA estimation.

MDA estimation

4.0 ml suspension

↓

2 ml Reagent II added

↓

centrifuged at 1,500 g for 10 mins

pellet → discarded

supernatant

↓

4.0 ml aliquot withdrawn into boiling tube

↓

1.0 ml Reagent III added

↓

incubated in boiling water bath with marble on top for 15 mins

↓

cooled immediately in cold water (centrifuged if turbid)

↓

spectrum scanned between 450-600 nm on Sp 800 using reagent blank*

* Reagent blank was prepared by adding 1.0 ml Reagent III to 4 ml of mixture made by mixing 2 ml 2 mM Na azide, 2 ml 20 mM H_2O_2 and 2 ml Reagent II. The mixture was boiled for 15 mins and cooled.

MDA concentration can be calculated by the following expression:

$$\text{MDA (n mol/g Hb)} = \frac{(O.D._{530} - O.D._{600}) \times 1 \times 10^9 \times 1/200}{\text{molar extinction coefficient} \times \text{Hb}}$$

$O.D._{530} - O.D._{600}$ - correct for yellow colour from sodium azide.

1×10^9 - converts M to nM

molar extinction coefficient of MDA = 1.56×10^5

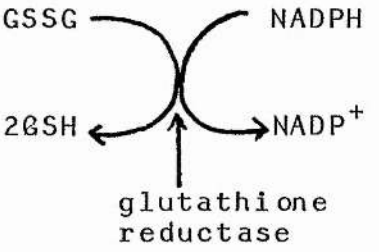
$1/_{200}$ - converts concentration of MDA to amount of MDA in the 5 ml mixture

Hb - g Hb in the 5 ml mixture

2.3.4 Glutathione Reductase (GR) Assay

2.3.4.1 Principle

Reaction:



Notes:

GSSG oxidized glutathione

GSH reduced glutathione

$O.D._{340}$ is monitored to detect the decrease of NADPH

2.3.4.2 Preparation of haemolysate (Beutler, 1977)

0.5 ml packed cells was mixed with 0.5 ml 0.9% NaCl and 9 ml stabilization solution (2.7 mM EDTA Na_2, pH 7.0 and 0.7 mM 2-mercaptoethanol). The stabilization solution is stable for several weeks stored at -20^0C. The haemolysates are stable for at least one year stored at -20^0C.

2.3.4.3 Reagents

1. Buffer

   0.5 M Tris-HCl/2.5 mM EDTA Na_2 pH 8.0

2. 9.9 mM neutralized GSSG

3. 1 mM NADPH

   since NADPH is more stable in alkaline solution, 10 fold diluted buffer was used to prepare the NADPH solution

4. 0.3 mM FAD (flavine adenine dinucleotide)

2.3.4.4 Procedure (Beutler, 1977)

Both glutathione reductase activity with and without FAD were determined.

Assay without FAD

Added in this order	Blank	Sample	Conc. in the cuvettes
Buffer	300 µl	300 µl	0.05 M Tris-HCl, 0.25 mM EDTA
Water	1.37 ml	1.37 µl	-
Haemolysate	30 µl	30 µl	-
Incubated at 37°C for 10 mins			
GSSG	(1 ml H_2O)	1 ml	3.3 mM
Incubated at 37°C for 10 mins			
NADPH	300 µl	300 µl	0.1 mM

Blanks and tests were assayed in duplicate.

Assay with FAD

Added in this order	Blank	Sample	Conc. in cuvettes
Buffer	300 µl	300 µl	0.05 M Tris-HCl 0.25 mM EDTA
H_2O	1.36 ml	1.36 ml	-
FAD	10 µl	10 µl	1 µM
Haemolysate	30 µl	30 µl	-
Incubated for 10 mins at 37°C			
GSSG	(1 ml H_2O)	1 ml	3.3 mM
Incubated for 10 mins at 37°C			
NADPH	300 µl	300 µl	0.1 mM

Duplicate cuvettes were assayed as before.

Glutathione reductase activity was expressed as µmoles of NADPH per min. per g. of haemoglobin, and was calculated from the following expression:

$$\text{GR activity } (\mu\text{moles/min./g Hb}) = \frac{\Delta O.D._{340} \times 1 \times 10^6 \times 1000}{\text{molar extinction coefficient} \times \text{Hb} \times 3}$$

molar extinction coefficient of NADPH = 6.22×10^3

Hb = haemoglobin in the 3 ml cuvette.

2.3.5 Catalase Assay

2.3.5.1 Principle (Abeliovich and Cohen, 1978)

O_2 evolved from H_2O_2 by the catalysis of catalase was measured by an oxygen electrode.

$$2H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2$$

2.3.5.2 Preparation of haemolysate

Haemolysate was prepared as described at 2.3.4.2, but diluted 5 times with stabilization solution before assay.

2.3.5.3 Procedure

A Clark oxygen electrode was used at 37°C.

1. Used saturated KCl to cover the O_2 electrode.
2. KCl solution and O_2 electrode covered with teflon membrane (without bubbles in the KCl solution).
3. Electrode set up by injecting water into the chamber, setting 30% of the chart as 100% air saturated H_2O. (O_2 electrode set up this way will detect O_2 levels above that of 100% air saturated water).
4. Sodium dithionite introduced into the chamber to absorb all the oxygen present to zero the electrode.
5. Water removed by suction, 3 ml of 30 mM H_2O_2 pipetted into the chamber and chart run until it gave a steady baseline.
6. Standard enzyme or haemolysate introduced by syringe and rate recorded.

2.3.5.3.1 Assay with commercial catalase

In order to investigate the linearity of the relationship between the rate of O_2 evolved and the amounts (units) of catalase added, commercial catalase (0.2 U, 0.4 U . . . 2.0 U in 3 µl) was used.

Since 100% air saturated H_2O at 37°c contains 6.77 mg O_2/l, the amount of O_2 evolved can be calculated.

2.3.5.3.2 Assay with haemolysate

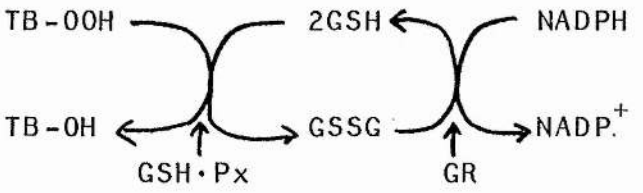
3 µl of 5 x diluted haemolysate was used to assay catalase activity which was expressed as mg O_2 evolved/ min./g Hb

2.3.6 Glutathione Peroxidase Assay (Beutler, 1977)

2.3.6.1 Principle

Hydrogen peroxide (H_2O_2) is the most abundant physiological substrate for GSH·Px but as it is relatively unstable and catalase will interfere with the result, an artificial substrate t-butyl hydroperoxide (TBH or TB-OOH) is commonly used. The reaction is linked to GR which oxidizes NADPH.

Reaction:



Notes:

TB-OH - tertiary butyl hydroxide

2.3.6.2 Reagents

1. Buffer

   1 M Tris-HCl/5 mM Na_2 EDTA pH = 8.0.

2. 6 mM GSH.

3. 1 mM NADPH Na_4 in 10 fold diluted buffer.

4. Glutathione reductase. (200 U/ml)

5. 21 mM TBH.

2.3.6.3 Procedure

Added in this order	Blank		Sample	Concentration in the curvettes
	1	2		
Buffer	300 µl	300 µl	300 µl	0.1 M Tris-HCl 0.5 mM EDTA
H_2O	1.075 ml	1.055 ml	1.045 ml	-
GSH	1 ml	1 ml	1 ml	2 mM
NADPH	600 µl	600 µl	600 µl	0.2 mM
GR	15 µl	15 µl	15 µl	3 U
Incubated 10 mins at 37°C				
Haemolysate	—	30 µl	30 µl	—
Incubated 10 mins at 37°C				
TBH	10 µl	—	10 µl	70 µM

Triplicate assays were done on each sample and blank. Activity of gluthione peroxidase can be calculated from the following expression:-

$$\text{GSH-Px } (\mu\text{moles NADPH/min./g Hb}) = \frac{\left[\Delta O.D._{340} \text{ in sample} - (\Delta O.D._{340} \text{ in Blank 1} + \Delta O.D._{340} \text{ in Blank 2})\right] \times {}^{3}/_{1000} \times 10^{6}}{\text{molar extinction coefficient} \times Hb}$$

Notes:

molar extinction coefficient of NADPH = 6.22×10^{3}

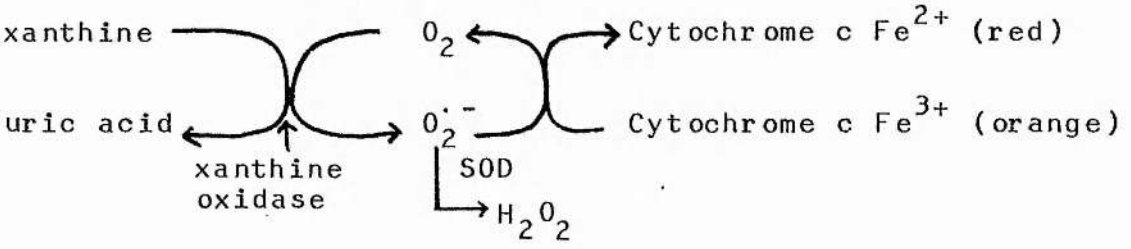
10^{6} = converts M to µM

Hb = g Hb in the 3 ml cuvette

2.3.7 Superoxide Dismutase (SOD) Assay

2.3.7.1 Principle (Crapo, McCord and Fridovich, 1978)

The substrate of SOD is the unstable superoxide free radical ($O_2^{\cdot -}$). As it is rapidly broken down, even if it were possible to add $O_2^{\cdot -}$ at the start of the assay the concentration in the assay will be unknown and constantly changing. An indirect assay was used instead. Superoxide is generated at a constant rate by xanthine oxidase, converting xanthine into uric acid. Cytochrome c was used as a detection reagent for $O_2^{\cdot -}$.



Superoxide free radical acts as a reducing agent. As it has a single unpaired electron and a negative charge, it loses the electron easily due to repulsion of like charges.

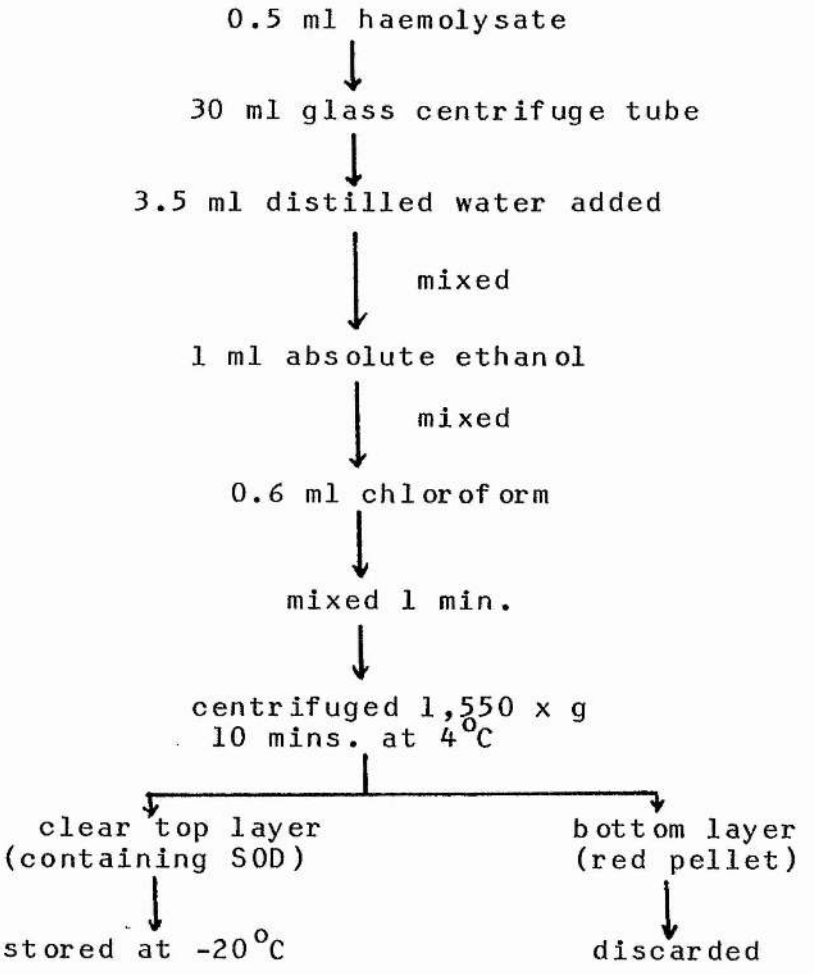
Increase in $O.D._{550}$ of cytochrome c as it is reduced at a constant rate can be measured. If SOD is added, the reduction of cytochrome c will be inhibited and this rate decreased. The change can be calculated as a % inhibition which over a certain finite range will increase in proportion to the amount of SOD added.

2.3.7.2 Preparation of SOD extract (Winterbourn et al., 1975)

1 ml of washed packed cells were haemolyzed by the addition of 1.5 ml distilled water. The haemoglobin concentration of the haemolysate was adjusted to 10 g/100 ml when possible. If the haemoglobin concentration was much less than 10 g/100 ml, more packed red cells were added to the haemolysate and the haemoglobin concentration re-

calculated and adjusted to 10 g/100 ml.

Haemoblogin interferes with the SOD assay and must be removed. This is done by chloroform-ethanol precipitation.



All reagents were ice cold and mixed as quickly as possible.

2.3.7.3 Reagents

1. Buffer

50 mM Na_2CO_3
10 µM KCN
0.1 mM EDTA

adjusted to pH 10.0 by adding 50 mM $NaHCO_3$

Modifications:

(i) Original method used pH 7.8 in K_3PO_4 buffer, but

assaying at pH 10.0 can increase sensitivity.

(ii) KCN abolishes interference from cytochrome oxidase. This concentration gives 100% inhibition of cytochrome oxidase without affecting SOD. The buffer is stable at $4^{o}C$ for several weeks.

2. Xanthine

1.0 mM in buffer

This must be heated to dissolve the xanthine. Xanthine concentration was increased from 0.5 mM in the original method to 1.0 mM to increase sensitivity.

3. Ferricytochrome c

0.1 mM in buffer

Commercially available cytochrome c contains some water and ferrocytochrome c as well as ferricytochrome c. It was necessary to calculate spectrophotometrically in a more concentrated solution then dilute to 0.1 mM. Approximately 1.0 mM cytochrome c (in buffer) was diluted 30 fold in a cuvette and $O.D._{550}$ read (X). Then a few crystals of sodium dithionite was added tocompletely reduce the cytochrome. $O.D._{550}$ was again read (Y). The true concentration of ferricytochrome c can now be obtained from the following expression:-

$$[\text{ferricytochrome c}] = \frac{\text{dilution factor } (Y-X)}{\text{molar extinction coefficient}}$$

dilution factor = 30

molar extinction coefficient = 21,000

Once the true concentration of ferricytochrome c is known, this can be diluted to 0.1 mM. All dilutions of cytochrome c were done in buffer. The xanthine and

cytochrome c are stable at -20°C for several weeks.

4. Mixture A

10 ml xanthine and 10 ml cytochrome c were made up to 100 ml with buffer. It is stable for several weeks at -20°C.

5. Xanthine Oxidase

Commercial xanthine oxidase is supplied as a suspension in $(NH_4)_2SO_4$, 28 U/ml. The enzyme suspension was diluted with distilled water such that 30 µl catalyzed a change at $O.D._{550}$ of 0.02/min. due to reduction of cytochrome c. The dilution factor should be adjusted to give 0.02± 0.002 units at $O.D._{550}$. This blank rate had to be checked regularly during the assay (at least once per working day) as the xanthine oxidase loses activity over a period of time in dilute solution. The precise value of blank rate is critical since a small change can lead to a large error in the calculation of % inhibiton.

2.3.7.4 Procedure (Crapo et al., 1978)

(i) Establishment of blank rate at 0.02 $O.D._{550}$ units/min

3.0 ml mixture A and 40 µl distilled water were added to a 3 ml cuvette and incubated at 37°C for 10 mins. 30 µl of xanthine oxidase (with appropriate dilution) was added and the $O.D._{550}$ monitored. If the rate was not 0.02±0.002 $O.D._{550}$ units/min, the xanthine oxidase

concentration was altered appropriately and the above repeated until 0.02±0.002 $O.D._{550}$ units/min. was achieved.

(ii) Preparation of calibration curve for SOD

Commercial SOD purified from bovine RBC (2900 U/mg) was made up to give standards of 0.5 µg/ml and 1.0 µg/ml from a more concentrated stock solution. To a 3 ml cuvette 3.0 ml mixture A and varying amounts (10-40 µl) of the above solutions (to give 0.005 µg SOD - 0.04 µg SOD) were added. The final assay volume before xanthine oxidase was added was always adjusted to 3.04 ml with distilled water. This was incubated at 37°C for 10 mins. 30 µl xanthine oxidase (with appropriate dilution to give a blank rate of 0.02±0.002 $O.D._{550}$ units per min.) was then added and $O.D._{550}$ monitored for several minutes. % inhibition was calculated from:-

Blank rate = X (∿ 0.02)

Rate with SOD or sample = Y

% inhibition = 1 - (Y/X) x 100

A calibration curve of % inhibition versus SOD concentration was prepared. This was linear up to 0.03-0.04 µg SOD. A calibration curve was set up at the beginning of each working day.

(iii) Assay SOD activity in crude SOD extracts from RBC

3 ml mixture A and 10-40 µl of SOD extract (diluted 10 times) were pipetted into a 3 ml curvette and incubated at 37°C for 10 mins. 30 µl xanthine oxidase was added and the rate of change of $O.D._{550}$ was measured.

A range of volumes were added to ensure % inhibiton is in the linear range of the calibration curve. In addition, it is important for an unknown sample to ensure that the % inhibition is linear with the amount of extract added since the linear range for a crude SOD sample was found to be slightly different from that for purified SOD standard (see results section). The SOD concentration of the sample can then be calculated using the calibration curve.

Results were calculated both as mg SOD/g Hb and mg SOD/ml packed cell.

2.3.8 Effect of Hyperbaric Oxygen (HBO) Treatment to the Lipid Peroxidation Protective Enzymes

Patients receiving HBO treatment were given 20 sessions, each of 90 mins, on consecutive days except that there was no treatment on the 7th and 14th days of the programme. Blood was taken from four patients (GE, HF, TG and MG) within 30 mins of their 20th treatment and also before the commencement of the programme. Patient LL had completed a 20 treatment course 1 week prior to providing the after-treatment sample but had also, immediately prior to venepuncture, just received a 90 minute "top-up" session in the chamber. HBO treatment consisted of air at 2 atmospheres and patients were in a sitting position for the treatment period.

The four enzyme (GR, GSH·Px, catalase and SOD) assays for the red blood cells from MS patients after HBO treatment were performed as described above, as was the peroxide stress test.

3. RESULTS AND DISCUSSION

3.1 Membrane Phospholipid Organization of RBC of MS Patients

The phospholipid class composition of RBC from MS patients was normal, as shown in Table 22 which is consistent with the previous report (Homa et al., 1980).

The transbilayer organization of phospholipid in MS patients was also found to be not significantly different from that of control, as shown in Table 23 and Fig. 20.

The phospholipid class composition of MS ghosts was also found to be similar to that of controls (Table 24) and to that of intact cells. When ghosts from both groups were treated with the enzyme, all of the glycerophospholipids underwent complete conversion to their corresponding lyso-derivatives, indicating that these phospholipids in the native membrane are fully available when access of the enzyme to both sides of the membrane is possible. The TLC patterns of the MS whole erythrocytes and ghosts and the enzyme-treated erythrocytes and ghosts are shown in Figs. 21, 22, 23 and 24.

Lipid asymmetry of the red cell membrane is thought to be maintained by the cytoskeleton (Marchesi, 1979), so that from these results, it can be suggested that there is no gross abnormality in the red cell cytoskeleton in MS patients.

Table 22 Phospholipid Class Composition of Whole Erythrocytes of MS Patients and Healthy Controls (expressed as lipid phosphorus w/w)

Phospholipids	Controls (n=12)	MS patients (n=6)
PC	30.97±2.78	29.11±2.10
SM	26.89±2.05	28.82±2.58
PS	10.75±1.04	10.16±2.44
PE	31.21±1.79	29.7±1.37
yield (µ mol lipid P/ml packed cells)	3.10±0.50	3.14±0.56
recovery %	89.85±8.70	88.85±7.50

Table 23 Degradation of Glycerophospholipids in Whole Red Cells Incubated with Bee Venom Phospholipase A_2

		% Total phospholipid class degraded ($\bar{X}$ ± S.D.)			Haemolysis % ($\bar{X}$ ± S.D.)
	n	PC	PE	PS	
Controls	6	51.73±7.76	6.24±4.23	0	1.04±0.68
MS Patients	6	59.86±7.00	2.85±2.79	0	1.24±0.50

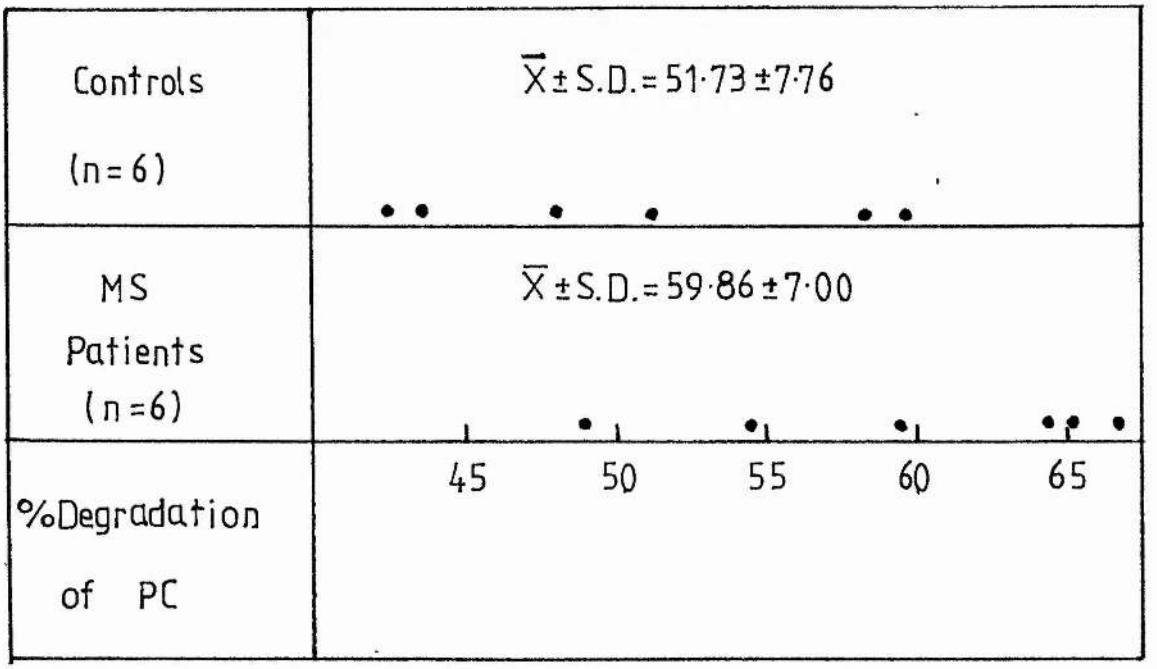


Fig. 20. Degradation of PC in Whole RBC in Controls and MS Patients.

Table 24 Phospholipid Class Distribution of Normal Ghosts and MS Ghosts (expressed as lipid phosphorus w/w)

Phospholipid	MS Ghosts (n=6)	Normal Ghosts (n=6)
PC	28.88±2.00	20.93±2.78
SM	29.07±2.30	26.46±2.90
PS	12.60±1.80	12.80±1.87
PE	28.00±1.90	29.43±1.30
yield (μ mol lipid P/ml packed cells)	2.62±0.40	2.93±0.54
recovery %	86.85±9.40	85.14±9.34

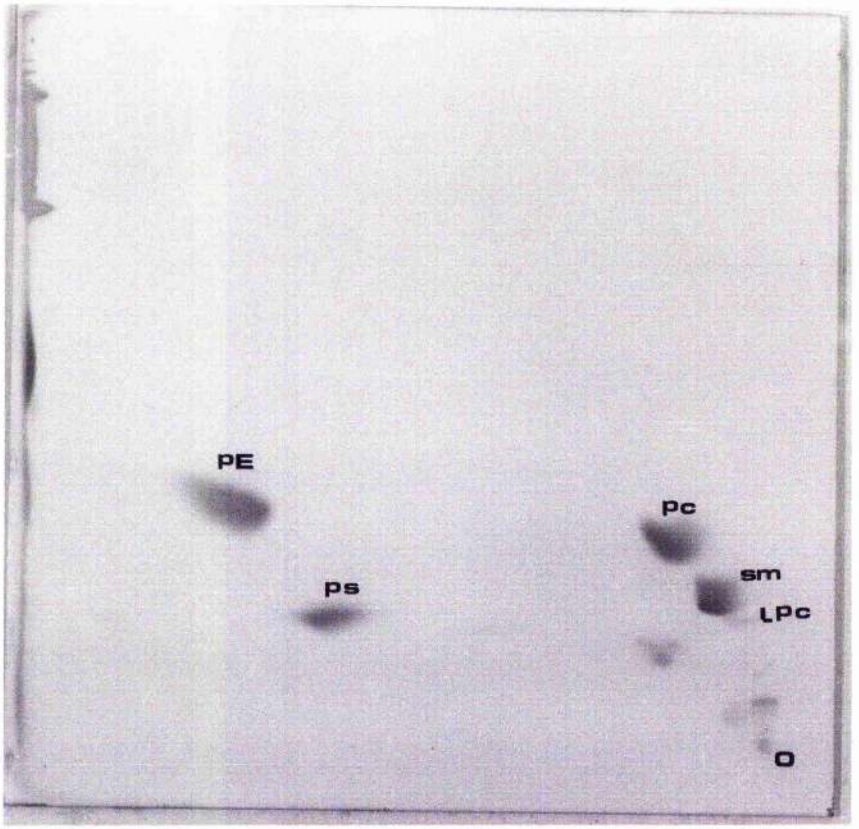


Fig. 21 Lipid Extracts of MS RBC. Loading: Lipids from 0.3 ml packed red cells.

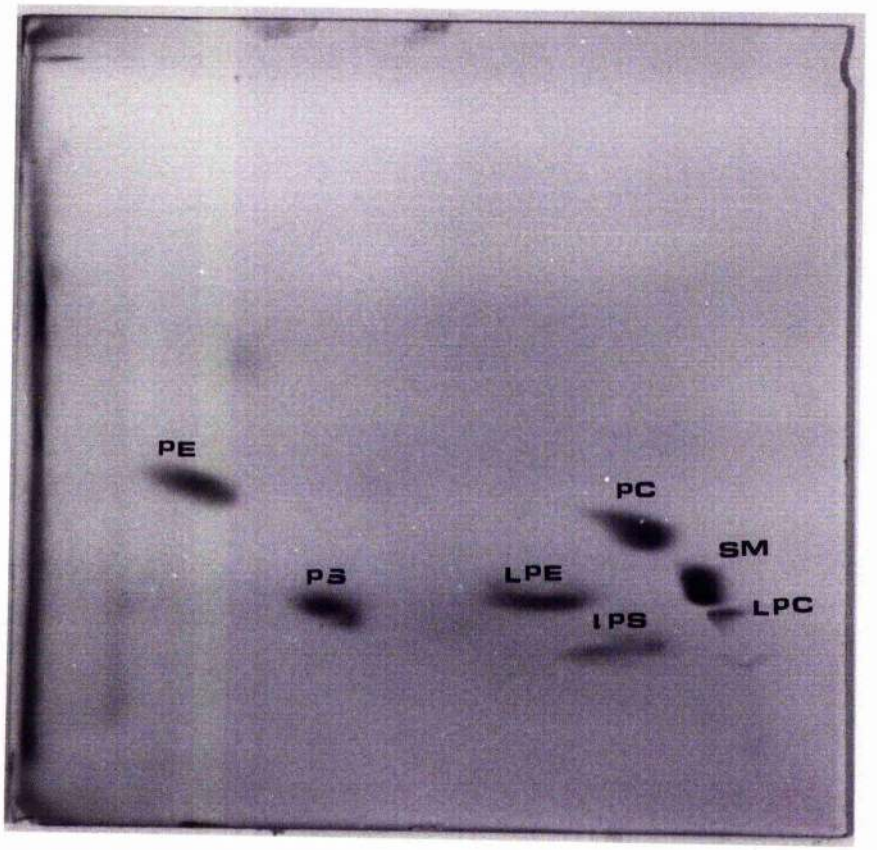


Fig. 22 Lipid extracts from MS ghosts. Loading: ghosts originated from 0.3 ml packed cells.

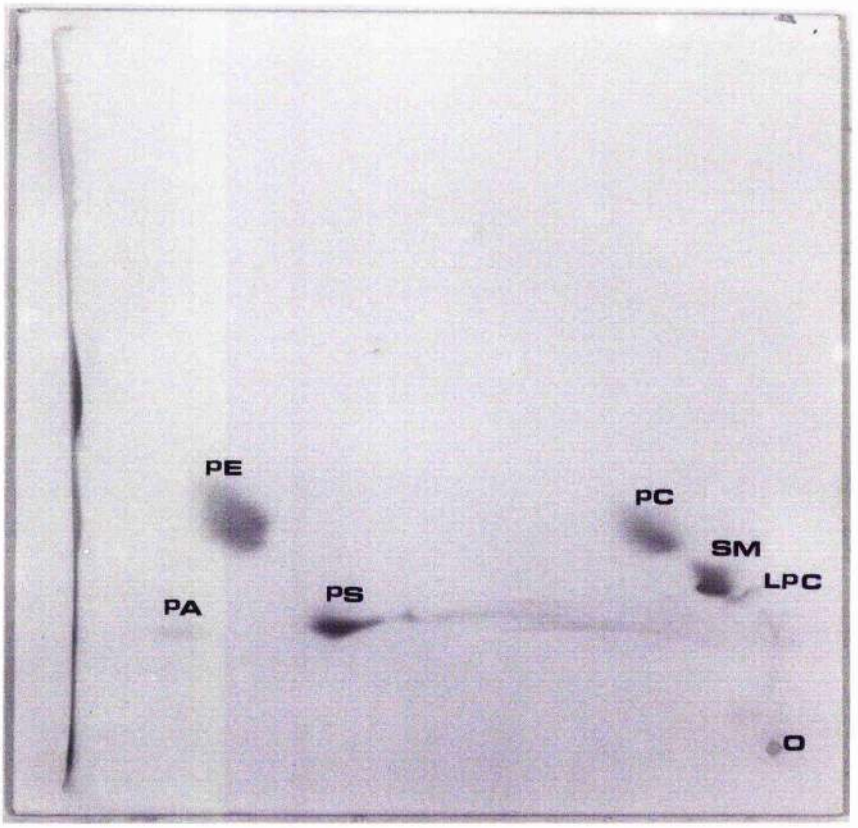


Fig. 23 Lipid extracts of MS RBC incubated with phospholipase A_2 (20 U/0.2 ml packed cells for 2 hrs at 37°C). Loading: Lipids from 0.3 ml packed cells.

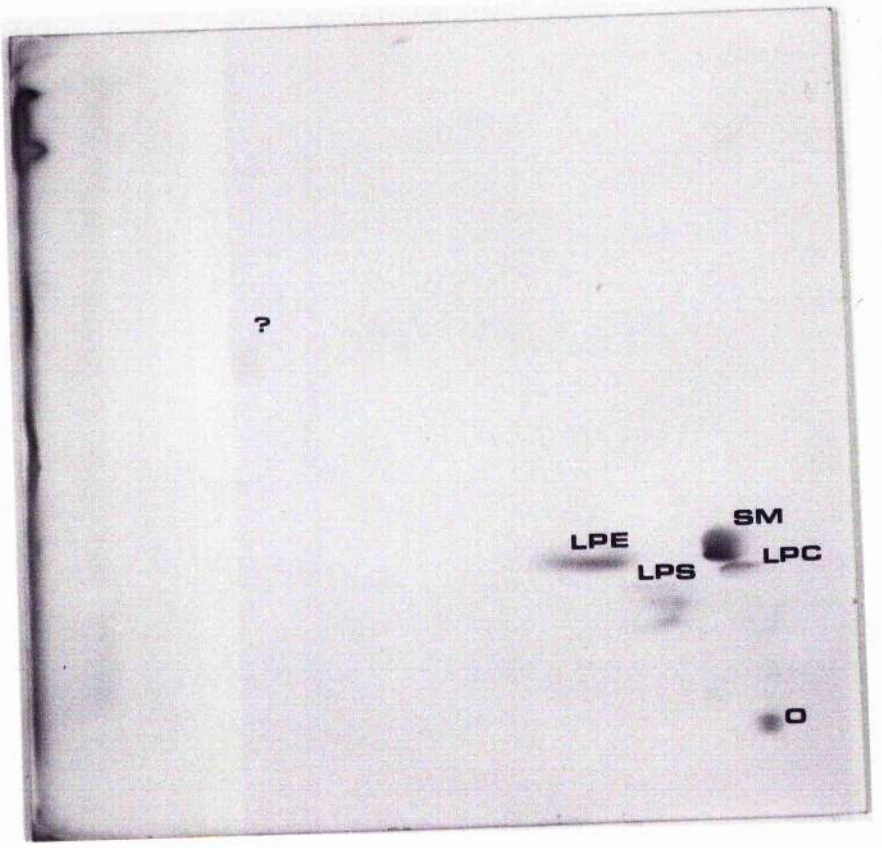


Fig. 24 Lipid extracts from MS ghosts incubated with phospholipase A_2 (20 U/0.2 ml packed cells for 2 hrs at 37°C). Loading: ghosts originated from 0.3 ml packed cell.

Szeinberg (1979) and Shukla et al. (1977) found decreased activity of red cell glutathione peroxidase in MS red cells. Another peroxidation protective enzyme, glutathione reductase, was also found to be slightly decreased by Jensen (1980). The normal phospholipid class distribution in MS erythrocytes found in this work indicates that the decreased activities of these two enzymes does not result in losses of phospholipids of the more unsaturated type, such as PE and PS, although the susceptibility to peroxidation of the MS RBC lipid might be expected to be increased.

Changes in membrane lipid organization have been suggested to be responsible for many of the changes found in the physical properties and membrane-bound enzymes in RBC, in conditions such as sickle cell anaemia (Lubin, Chiu, Bastacky, Roelofsen and van Deenen, 1981) and Duchenne muscular dystrophy (Chapter I of this work).

Increased red cell size (Prineas, 1968) and increased osmotic fragility (Stasiw et al., 1977; Schauf, 1980; Kurantsin-Mills, 1982) were found in MS erythrocytes. In addition, increased platelet stickiness was found in MS patients (Caspary et al., 1965). Prineas (1968) and Caspary et al. (1967) related this phenomenon with the change in size and fragility of MS red cells. They suggested the changes in those physical properties might imply change in the RBC membrane, probably related to phospholipids which induced leakage of ADP or other factors and these in turn, increased platelet stickiness. From

this work, it can be concluded that red cell-associated changes are therefore not due to alterations in lipid organization. In agreement with these findings, normal red cell deformability in MS patients demonstrated by Pollock et al. (1982) also implies a normal intrinsic membrane structure in MS erythrocytes.

Erythrocytes from MS patients have also been reported to exhibit reduced electrophoretic mobility when incubated with linolenic acid (Field et al., 1974; Field and Joyce, 1976), although this finding is rather controversial (Kurantsin-Mills, 1982) and there have been suggestions that this may be a property of MS plasma and an absorption phenomenon (Field and Joyce, 1982). This effect may be the result of a conformational rearrangement of charged group in the outer half of the bilayer induced by linoleate (Zukoski, Tamblyn, Swank and Seaman, 1979). If this is the case, it is also clear from the results of this work that the difference between normal and MS erythrocytes is not one of altered transbilayer phospholipid distribution.

3.2 Protective Mechanisms Against Lipid Peroxidation in RBC from MS Patients

3.2.1 H_2O_2 Stress Test

As shown in Fig. 25, the kinetics of MDA production are similar in both patients and controls, both of them show MDA production peaking and plateauing at 60 mins.

As shown in Table 25 and Fig. 26, the production of MDA is much decreased in MS patients compared with controls and the results are highly significant ($P < 0.001$, Student's t test).

This result suggests that MS red cells are better protected against oxidative challenge and lipid peroxidation than normals. One possible explanation is the vitamine E (an antioxidant and free radical scavenger,(Horwitt, 1965)). level in MS patients is higher than controls. Since this test, performed under the carefully controlled conditions of Stocks et al. (1971), correlates well, although not invariably with vitamin E status. It was considered possible that the MS patients had been receiving supplementary vitamin E, since many patients taking oils such as sun flower seed and evening primrose oil also take additional vitamin E. However after contacting all the patients in this study it was established that only one patient (TG) was receiving supplementary vitamin E. The activities of all four antioxidant enzymes in MS patients were found to be normal (results shown in Tables 26, 27, 28 and 29, Fig. 27, 28, 31 and 33). The increased resistance to peroxidation found in the peroxide stress test is therefore not due to

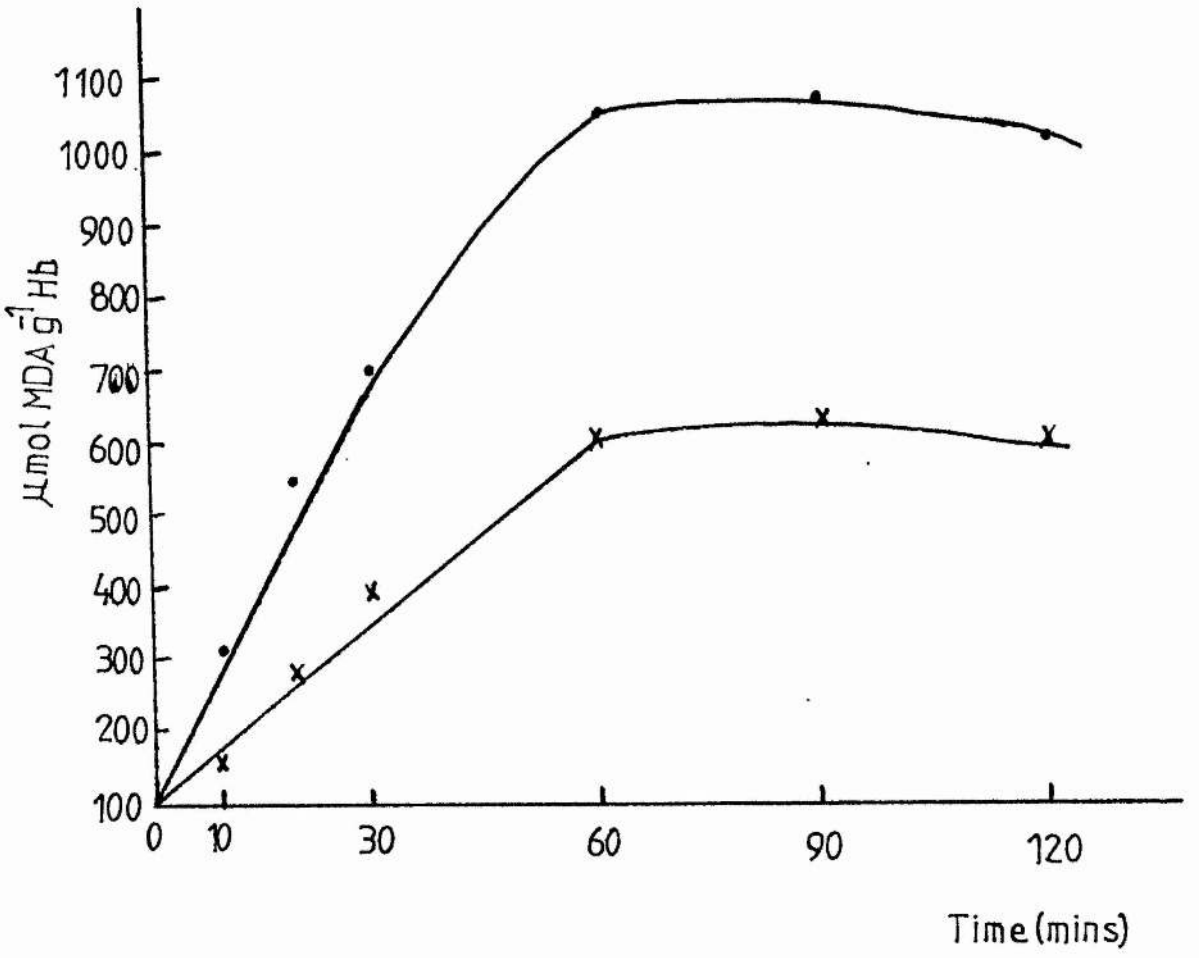


Fig. 25 H_2O_2 stress test.
nmol MDA produced per g of haemoglobin in a time course experiment.
control ·——· MS patient ✕——✕

Table 25 H_2O_2 Stress Test (Expressed as nmol MDA hr^{-1}/g^{-1} Hb in controls and MS Patients)

Controls (n = 13)		MS Patients (n = 11)	
1	1054.23	HF	675.23
2	942.92	NH	295.17
3	775.43	SMc	655.94
4	601.27	ID	204.98
5	939.40	LL	259.65
6	989.00	IA	302.15
7	733.20	PR	252.28
8	810.50	TG	254.06
9	861.10	HG	473.04
10	712.70	GE	649.13
11	747.70	MG	512.44
12	735.90		
13	643.90		
$\bar{X}$±S.D.	810.60±138.10	$\bar{X}$±S.D.	412.24±184.64

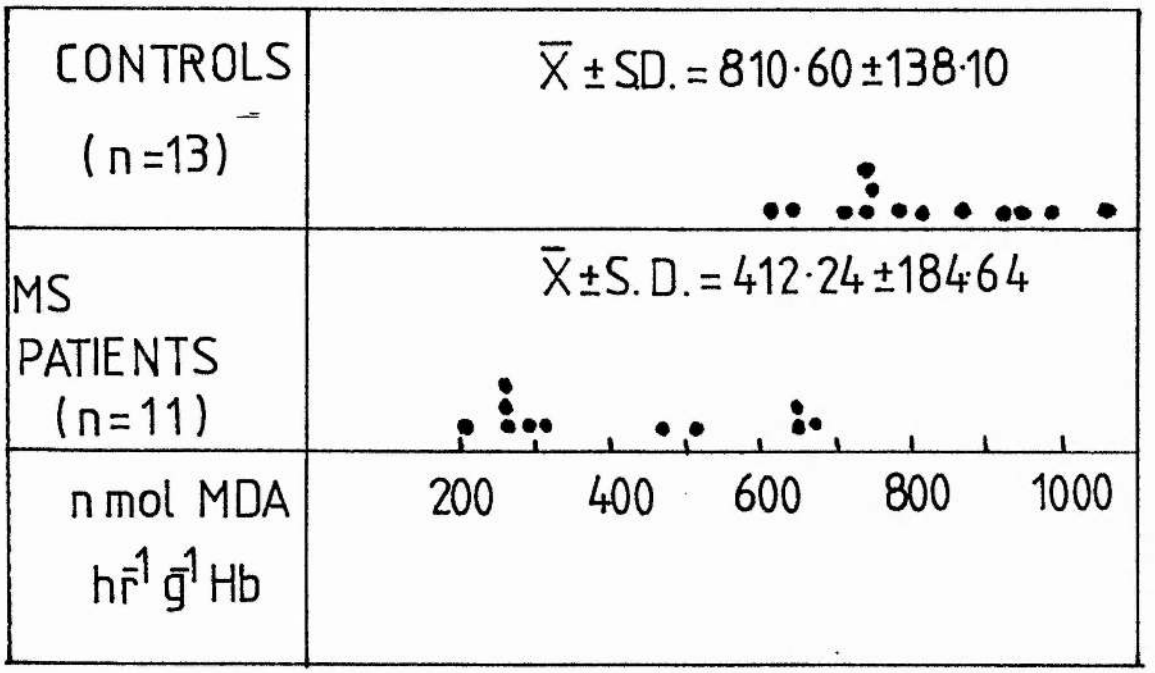


Fig. 26 H_2O_2 stress test (expressed as nmol MDA hr^{-1}/ g^{-1} Hb) in controls and MS patients.

increased activities of these enzymes but may be the consequence of elevated intracellular glutathione concentration or some other unrecognized antioxidant in the red cells. Further experiments which suggest themselves are to assay the concentration of glutathione, other antioxidants or vitamin E in the cells. Although vitamin E levels were found to be normal in MS patients in Finland (Wikström, Westermarck and Palo, 1976), different vitamin E levels may still be possibly found in British MS patients due to differences in race, diet and soil (Wikström et al., 1976). It is of interest that, although in their paper Wikström et al. conclude there is no significant difference in vitamin E concentrations, nevertheless the mean value quoted for MS patients is higher than that for controls. The resistance to peroxidative damage found in MS patients in this work may also be a property of MS plasma which was suggested to be the origin of the altered electrophoretic mobility of MS red cells in the presence of linoleic acid (Field and Joyce, 1982; Seaman et al., 1980). The experiments, suggested above may also be applied to plasma. Another area worthy of investigation would be to modify the conditions of the stress test (e.g. use a different oxidant stressor, such as dialuric acid) so as to produce a clear cut difference between MS patients and controls and perhaps develop it into a diagnostic test.

3.2.2 Antioxidant Enzyme Activities

3.2.2.1 Glutathione peroxidase (GSH·Px)

The GSH·Px activities for MS patients and controls are shown in Table 26 and Fig. 27.

The difference between normal and MS patients in red cell GSH·Px activities was non-significant. This finding is in contrast to the work of others (Shukla et al., 1977; Szeinberg et al., 1979) who found a significant reduction in this enzyme in MS, using the same substrate (tert-butyl hydroperoxide) as in this assay. The control values reported in the Shukla et al. (1977) article are extremely low (8.3±2.4) compared with others in the literature and those present here (21.81±4.30) which may suggest metholo-gical shortcomings. The reason for this discrepancy is not clear but may be due to population difference since in the study of Shukla et al. (1977) patients and controls were all Danish while the subject of Szeinberg et al. (1979) were all Israeli Jews.

3.2.2.2 Glutathione Reductase (GR)

Results are shown in Table 27 and Fig. 28.

GR activity of MS red cells was normal as compared to controls in the presence and absence of exogeneous FAD which is a cofactor of this flavoprotein enzyme. This finding is in contrast with that of Jensen et al. (1980) who found a slight decrease in GR activity in red cells of patients with MS. This enzyme is important for recycling reduced glutathione (GSH) which is converted to oxidized glutathione (GSSG) by GSH·Px. The fact that addition of

Table 26 GSH·Px Activities of Normal Controls and MS Patients (expressed as µmol NADH $min.^{-1}$ g^{-1} Hb)

Controls (n = 7)		MS Patients (n = 14)	
1	23.42	GE	20.09
2	17.57	HF	17.98
3	25.29	NH	12.90
4	27.73	MH	26.22
5	20.77	HG	24.79
6	22.56	ID	28.06
7	15.35	SMcL	20.35
		PR	19.87
		ED	19.58
		LL	19.03
		IA	19.44
		TG	14.48
		MG	12.50
		AT	10.85
$\bar{X}$±S.D.	21.81±4.30	$\bar{X}$±S.D.	18.88±5.30

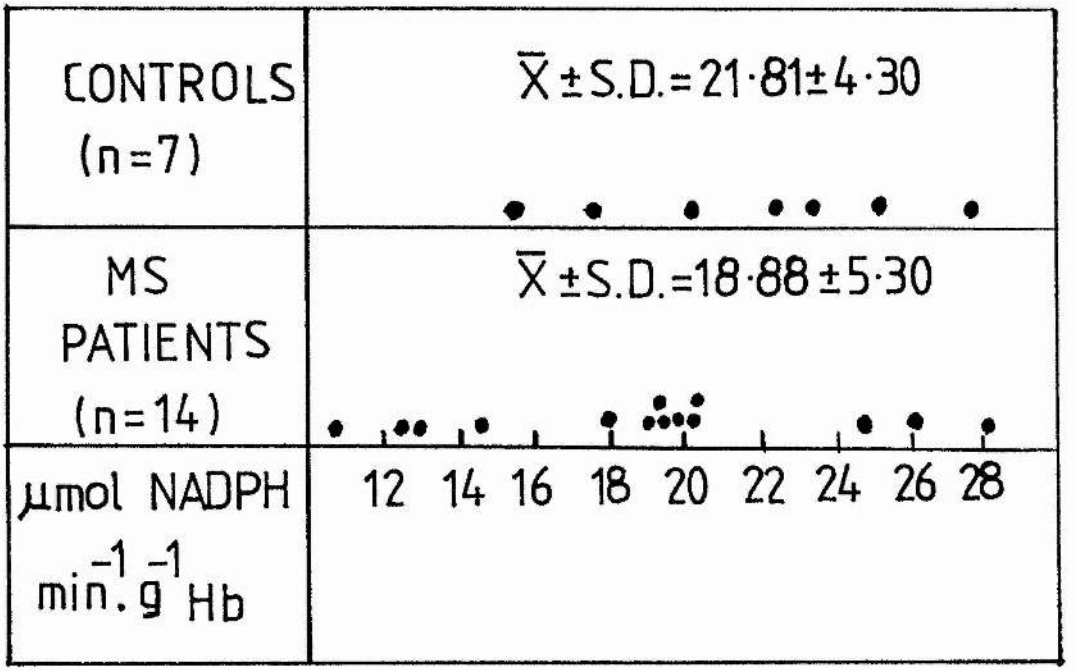


Fig. 27 GSH·Px activities of normal and MS patients.

Table 27 GR Activities of Controls and MS Patients

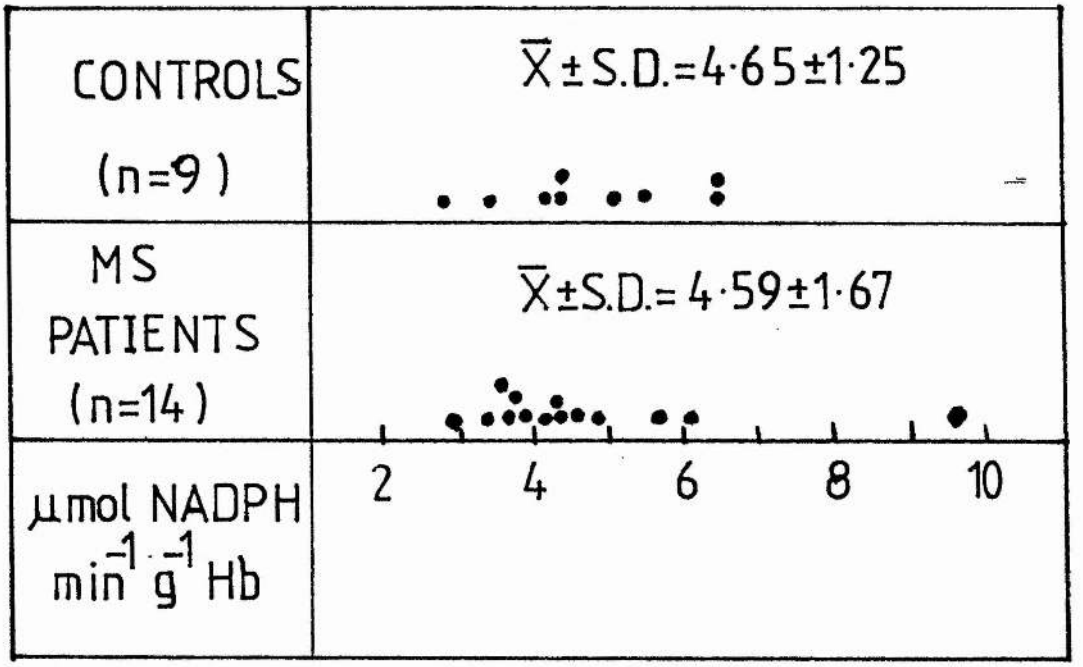
(i) Without FAD (expressed as μmol NADH min^{-1} q^{-1} Hb

Controls (n = 9)		MS Patients (n = 14)	
1	4.22	SMcL	3.36
2	5.00	PR	4.86
3	2.76	NH	4.10
4	5.39	LL	6.02
5	6.38	MG	3.86
6	4.23	GE	3.63
7	6.38	HF	2.92
8	3.39	HG	3.73
9	4.12	ID	9.58
		MH	4.24
		AT	3.51
		ED	5.68
		TG	4.52
		IA	4.27
$\bar{X}$±S.D.	4.65±1.25	$\bar{X}$±S.D	4.59±1.67

(ii) with FAD

Controls (n = 9)		MS Patients (n = 14)	
1	5.96	SMcL	5.09
2	5.68	PR	5.58
3	4.23	NH	4.69
4	6.43	LL	7.81
5	8.76	MG	5.49
6	5.06	IA	6.31
7	7.63	GE	5.98
8	4.48	HF	4.56
9	5.44	HG	4.90
		ID	11.77
		MH	4.64
		AT	5.17
		ED	7.197
		TG	5.76
$\bar{X}$±S.D.	5.96±1.46	$\bar{X}$±S.D.	6.07±1.89

(i)WITHOUT FAD



(ii) WITH FAD

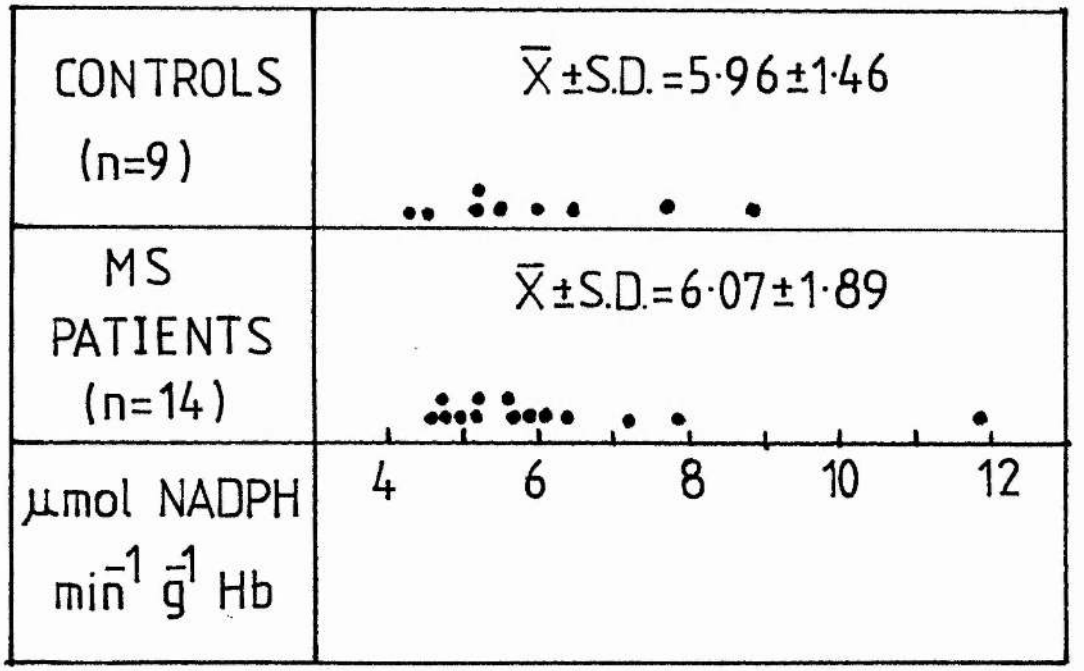


Fig. 28 GR activities of controls and MS patients.

FAD results in the same degree of stimulation of GR in MS patients and controls suggests that deficiency of riboflavin or its metabolism is not a feature of MS, as it is in some other disease states (Beutler, 1974).

3.2.2.3 Catalase

The linearity of the O_2 electrode assay was investigated using increasing amounts of commercial catalase and haemolysate. Fig. 29 and 30 show that the evolution of O_2 is linear up to 2.0 units of catalase and 10 µl of undiluted haemolysate. All subsequent assays were carried out within this linear range. Infact, 3 µl of 5 x diluted haemolysate were normally used so that O_2 evolved was in direct proportion to the amount of catalase present. Tabel 28 and Fig. 31 show the results for controls and MS patients and no significant difference was found between the two groups.

3.2.2.4 Superoxide dismutase (SOD)

Fig. 32 shows that the linear range for a crude SOD sample (up to about 60% inhibiton) was different from that for purified SOD standard (up to about 40% inhibiton), and this was taken into account when assaying samples. Because this assay is extremely sensitive to minor changes in conditions, a calibration curve was constructed once per working day.

SOD activities of MS patients and controls is shown in Table 29 and Fig. 33.

When SOD activity was expressed as mg SOD/ml packed cells, the SOD activity of MS patients was found to be significantly decreased ($P < 0.05$, Student's t test)

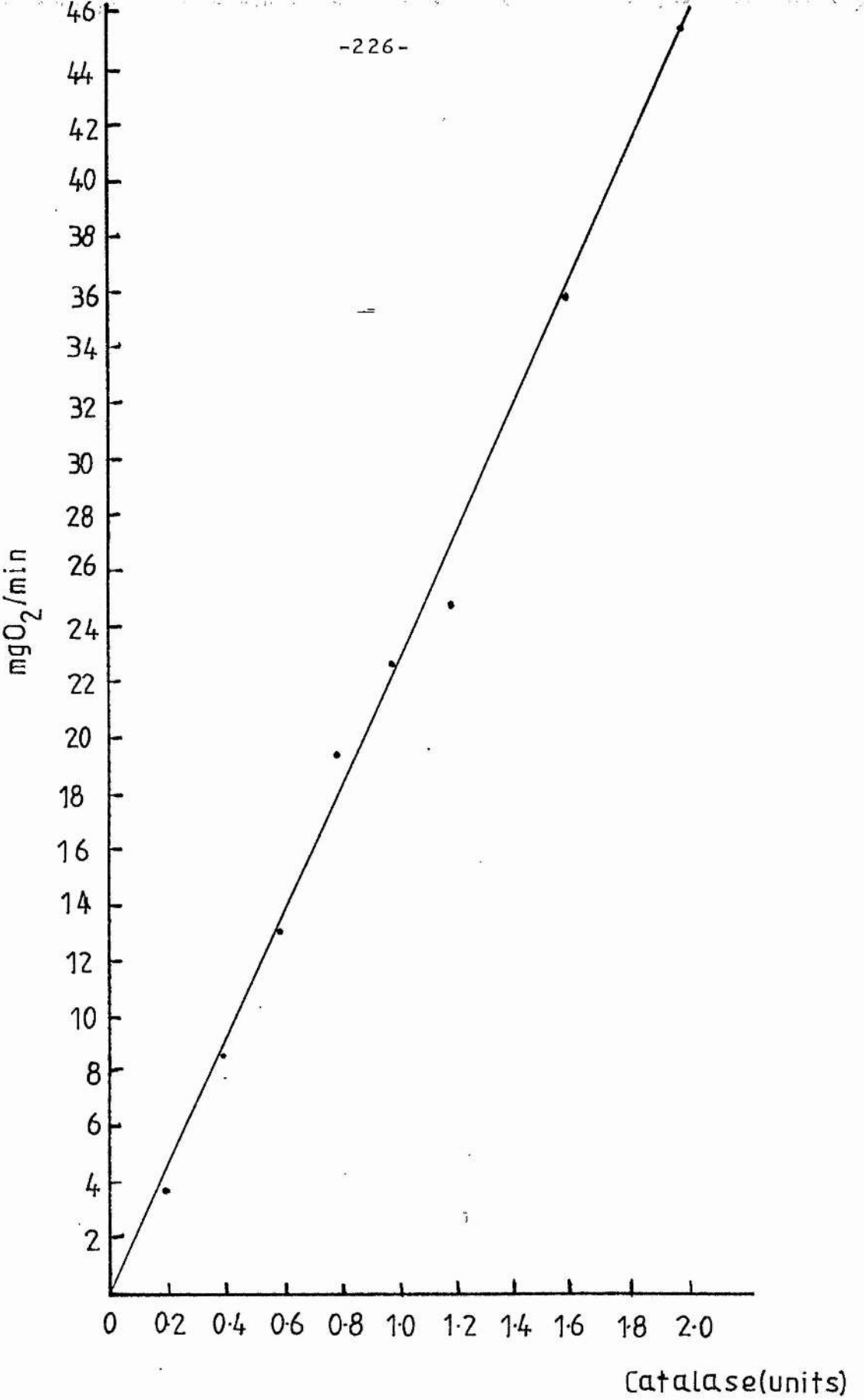


Fig. 29. mg O_2/min. evolved with commercial catalase.

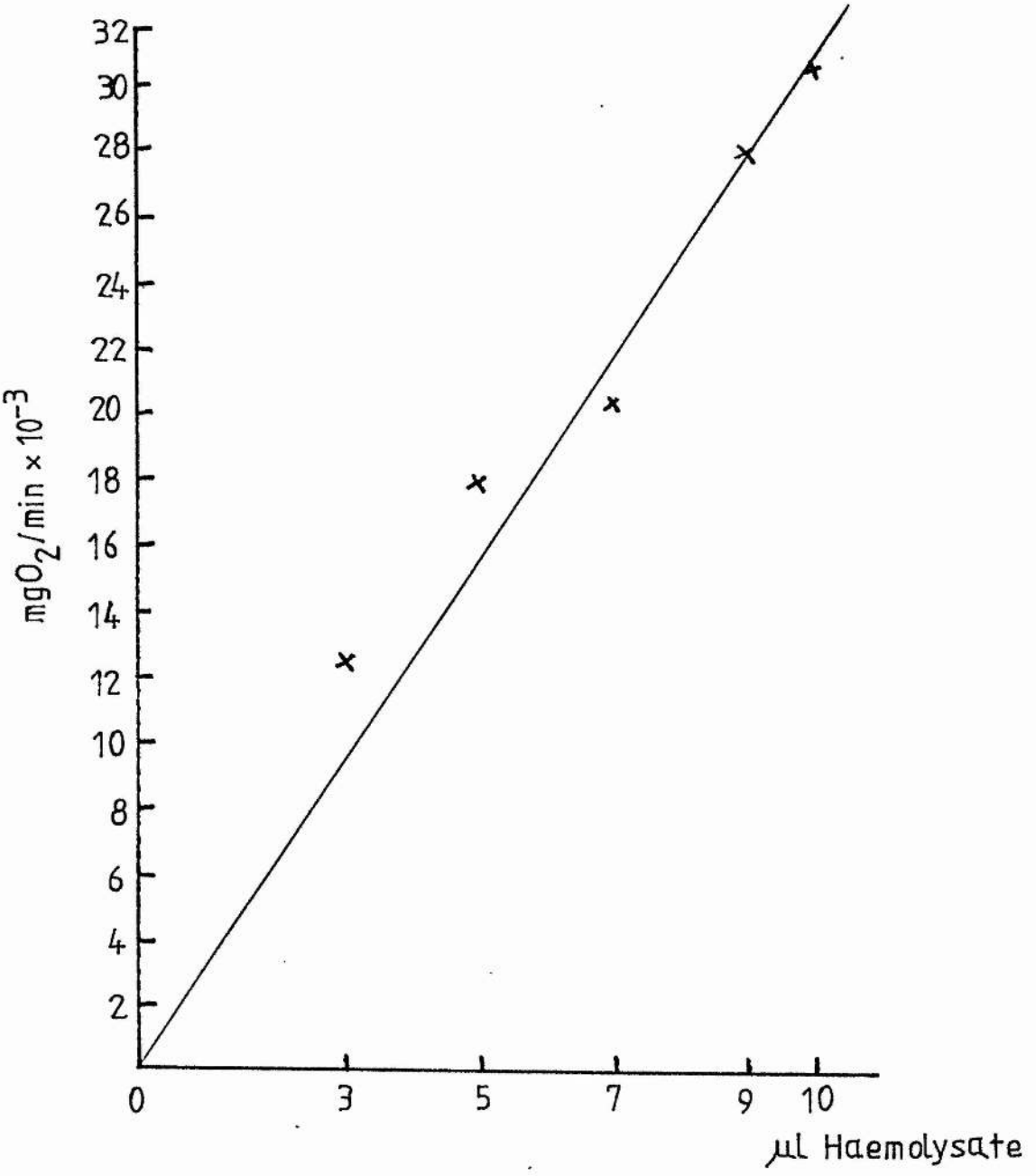


Fig. 30 mg O_2/min. evolved with haemolysate from normal red blood cells.

Table 28 Catalase Activities of Controls and MS Patients (expressed as μgO_2 min^{-1} g^{-1} Hb)

Controls (n = 8)		MS Patients (n = 14)	
1	1.8569	NH	1.0092
2	1.3256	LL	2.2323
3	2.6116	MG	0.6965
4	1.9243	HF	0.5912
5	1.0843	AT	1.1776
6	1.1516	GE	2.0919
7	0.4409	ED	0.9039
8	0.8178	HG	2.4096
		SMcL	1.2490
		PR	0.9262
		IA	2.4972
		ID	1.2974
		MH	0.9873
		TG	1.5449
$\bar{X}$±S.D.	1.4016±0.6900	$\bar{X}$±S.D.	1.4010±0.6569

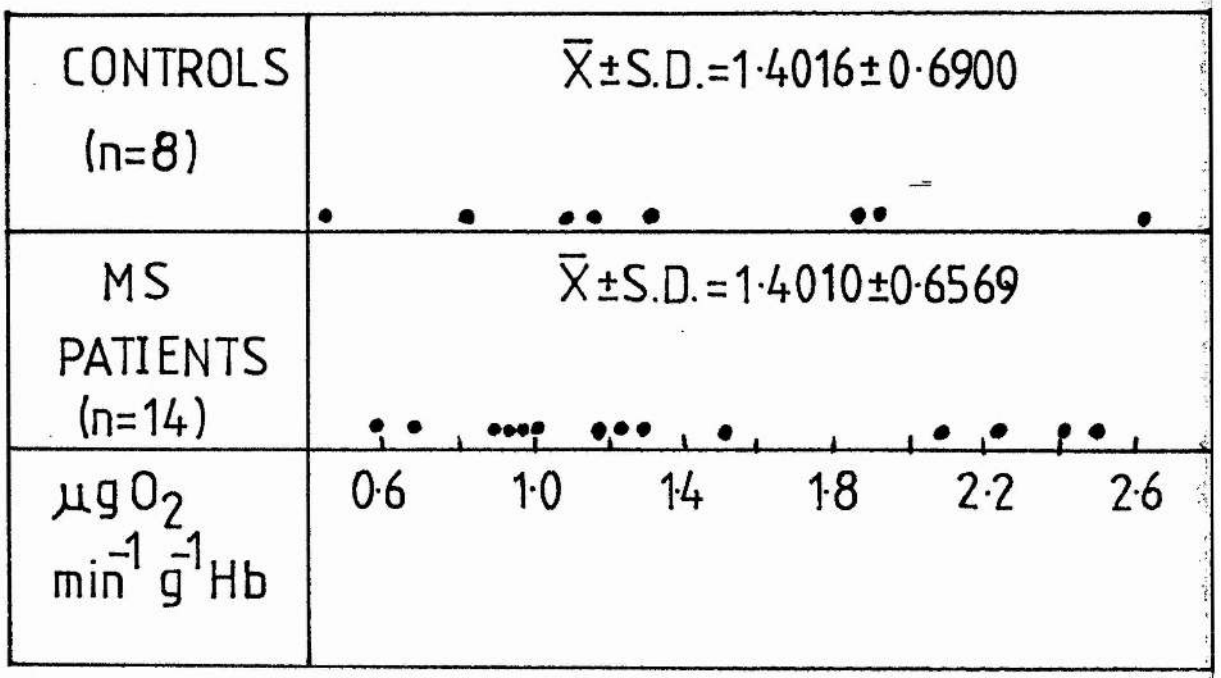


Fig. 31 Catalase activities of controls and MS patients.

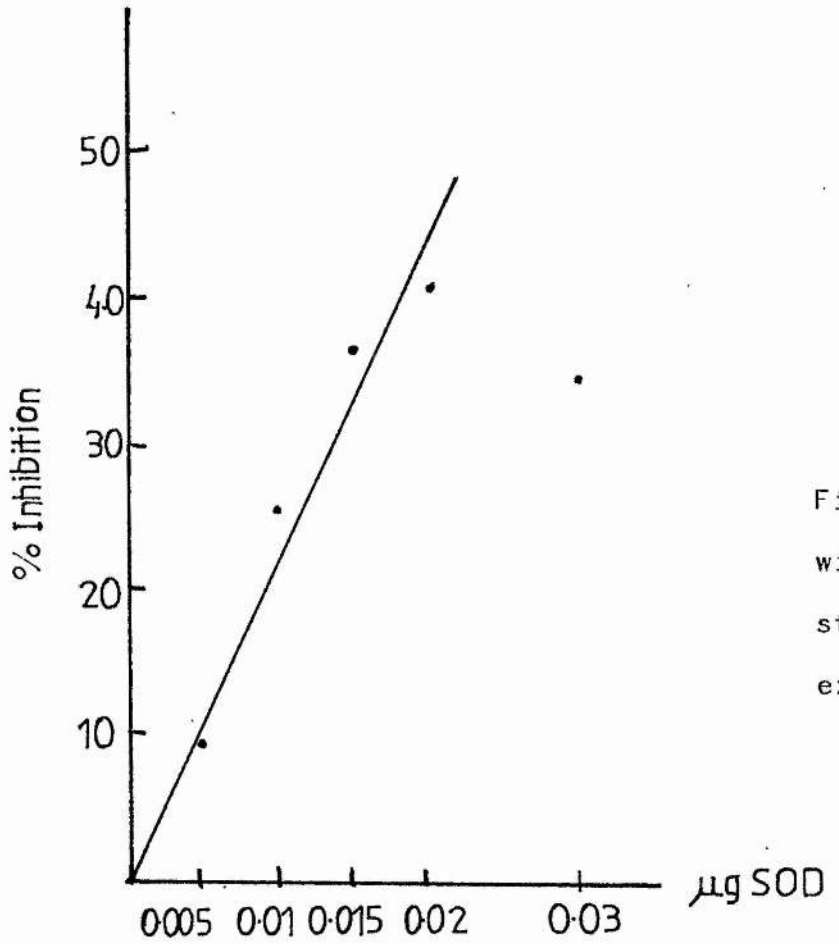


Fig. 32 % inhibition with purified SOD standard and SOD extract.

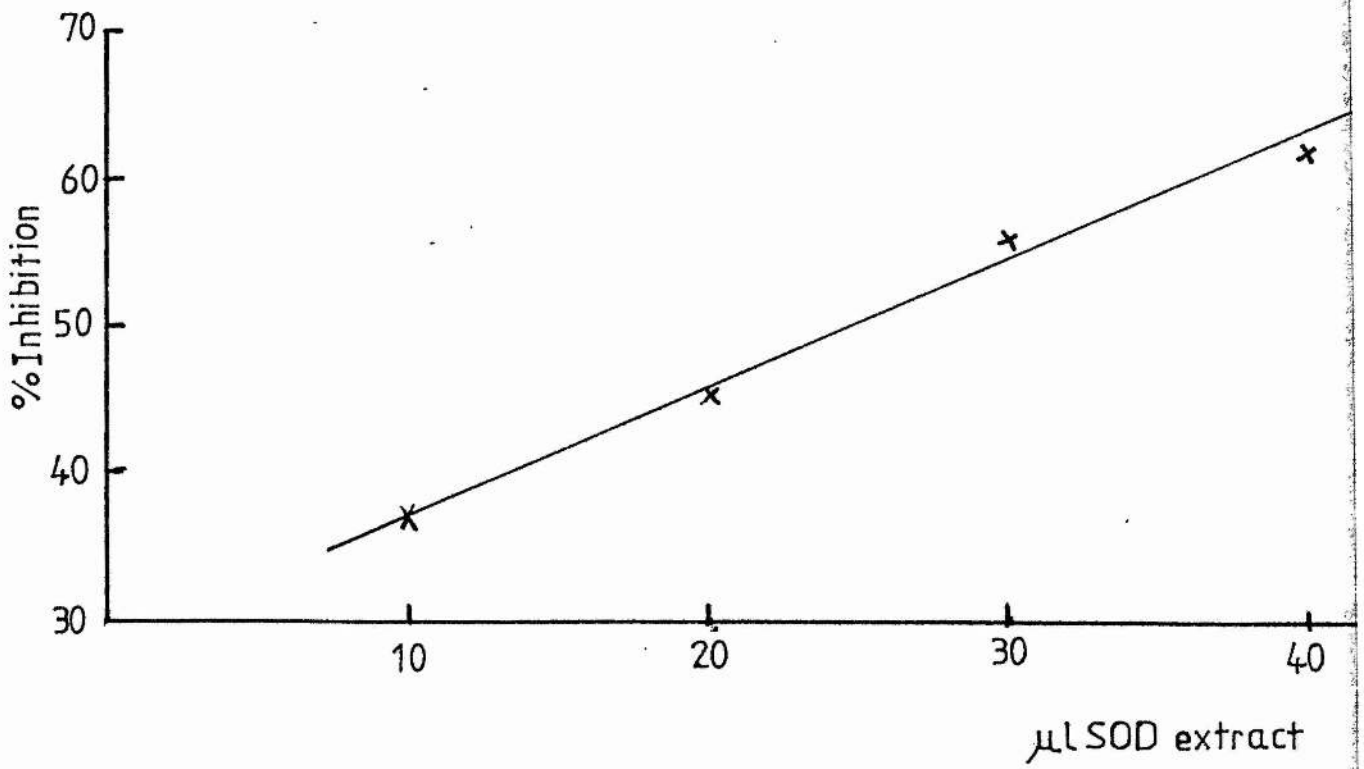


Table 29 SOD Activities of Normal and MS Patients

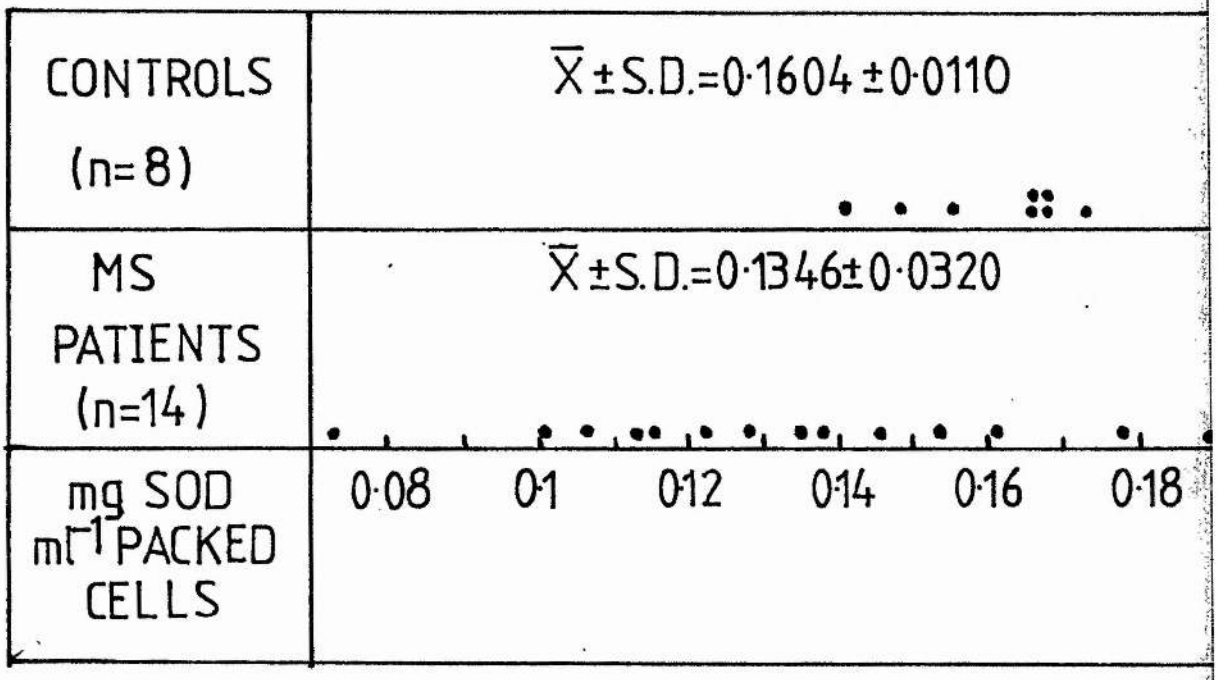
(i) Expressed as mg SOD ml^{-1} Packed Cells

Controls (n = 8)		MS Patients (n = 14)	
1	0.1403	NH	0.1286
2	0.1675	LL	0.1581
3	0.1676	AT	0.1774
4	0.1485	ID	0.1109
5	0.1657	HF	0.1989
6	0.1552	MG	0.1457
7	0.1659	TG	0.1141
8	0.1725	GE	0.1611
		ED	0.1369
		HG	0.0734
		SMcL	0.1156
		PR	0.1228
		IA	0.1354
		MH	0.1058
$\bar{X}$±S.D.	0.1604±0.0110	$\bar{X}$±S.D.	0.1346±0.0320

(ii) Expressed as mg SOD g^{-1} Hb

Controls (n = 8)		MS Patients (n = 14)	
1	0.5983	NH	0.4570
2	0.5606	LL	0.5688
3	0.6806	AT	0.5399
4	0.6665	ID	0.6838
5	0.6283	HF	0.8684
6	0.6304	MG	0.5682
7	0.6040	TG	0.2741
8	0.4981	GE	0.6702
		ED	0.4198
		HG	0.2489
		SMcL	0.4108
		PR	0.4505
		IA	0.5738
		MH	0.3235
$\bar{X}$±S.D.	0.6090±0.0586	$\bar{X}$±S.D.	0.5041±0.1706

(i)



(ii)

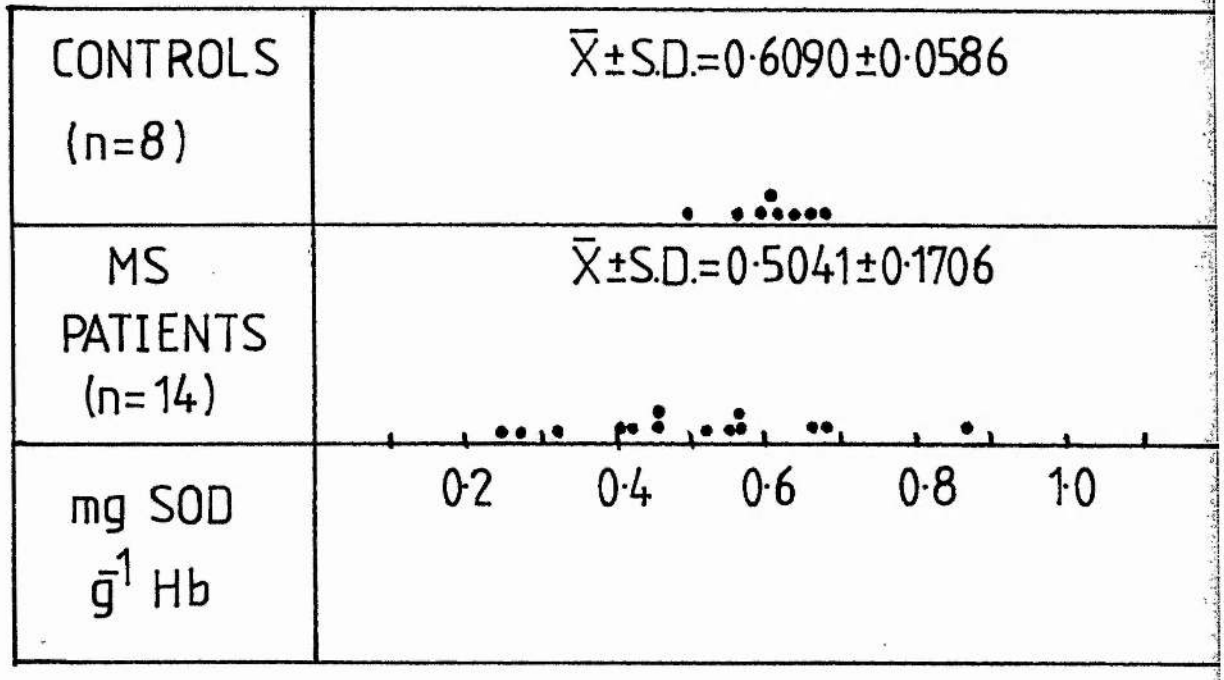


Fig. 33 SOD activities of controls and MS patients.

when compared to controls. But the difference became non-significant when the SOD activity was expressed as mg SOD/g Hb, and no differences in mean cell haemoglobin content between patients and control groups was found. Calculation of SOD activity will be more accurate when expressed on the basis of haemoglobin content rather than on the basis of volume of packed cells, since the volume of packed cells may vary with the rate of centrifugation and the completeness of the removal of saline after the final wash.

The activities of the four most important erythrocyte enzymes (SOD, catalase, GR, GSH·Px) involved in the destruction of $O_2^{\cdot -}$ and H_2O_2 are found to be unimpaired in the disease. It can be therefore concluded that if there is increased lipid peroxidation damage in the red cells in MS, this is not due to defective enzymic protection. However, in view of the reduced peroxidisability of MS red cells it seems unlikely that such damage is the cause of other abnormalities, such as increased fragility (Stasiw et al., 1977; Schauf et al., 1980; Kurantsin-Mills et al., 1982) and increased cell size (Prineas, 1968) found in MS erythrocytes. Although the activities of four protective antioxidant enzymes are found to be normal in MS red cells, it is still worthwhile to investigate the activities of these enzymes in CNS, since these antioxidant protective mechanism may still be impaired CNS.

3.2.3 Effects of HBO treatment on antioxidant protective mechanisms

Table 30 GR Activity of MS Patients Before and After HBO Treatment (expressed as μmol NADH min^{-1} g^{-1} Hb) (n = 5)

(i) without FAD

Patients	Before HBO Treatment	After HBO Treatment
LL	6.0155	4.3556
MG	3.8570	3.8184
GE	3.6257	4.2537
HF	2.9155	2.6520
TG	4.2726	6.7993
$\bar{X}$±S.D.	4.1859±1.1700	4.3758±1.5141

(ii) with FAD

Patients	Before HBO Treatment	After HBO Treatment
LL	7.8135	7.4321
MG	5.4884	5.2802
GE	5.9856	7.3106
HF	4.5562	5.0461
TG	5.7617	7.8368
$\bar{X}$±S.D.	5.9210±1.1890	6.5811±1.3100

Table 31 H_2O_2 Stress Test of MS Patients Before and After HBO Treatment (expressed as nmol MDA hr^{-1} g^{-1} Hb) (n = 2)

Patients	Before HBO Treatment	After HBO Treatment
TG	245.06	357.80
GE	649.13	409.97
$\bar{X}$±S.D.	451.59±279.35	383.88±36.88

Table 32 GSH·Px Activity of MS Patients Before and After HBO Treatment (expressed as μmol NADH min^{-1} g^{-1} Hb) (n = 5)

Patients	Before HBO Treatment	After HBO Treatment
GE	20.0905	16.1434
HF	17.9795	27.1446
LL	19.0305	25.9634
TG	14.4781	20.9586
MG	12.5044	16.8538
$\bar{X}$±S.D.	16.8166±3.2029	21.4127±5.0576

Table 33 SOD Activity of MS Patients Before and After HBO Treatment (n = 5)

(i) Expressed as mg SOD ml^{-1} Packed Cells

Patients	Before HBO Treatment	After HBO Treatment
LL	0.1581	0.0585
HF	0.1988	0.1860
MG	0.1457	0.0886
TG	0.1141	0.1525
GE	0.1611	0.1780
$\bar{X}$±S.D.	0.1555±0.0305	0.1327±0.0563

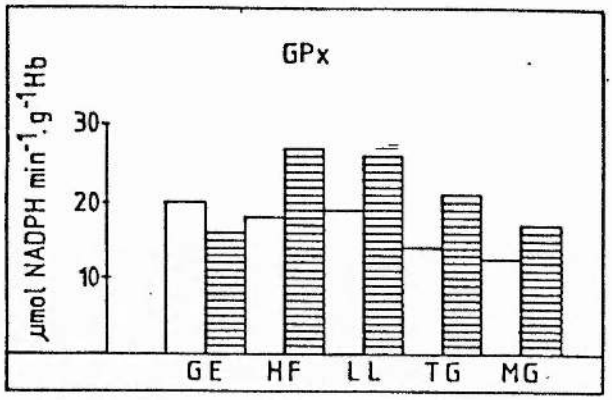
(ii) Expressed as mg SOD g^{-1} Hb

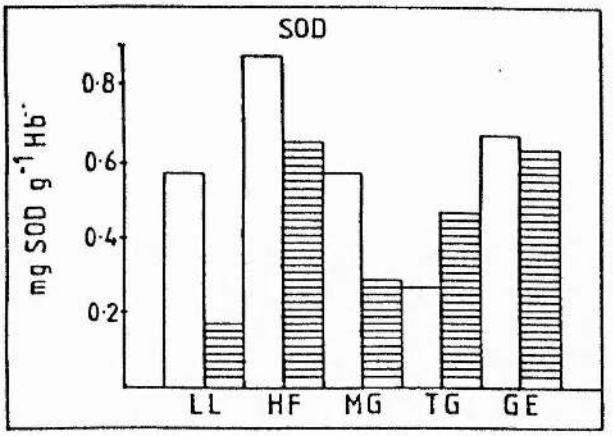
Patients	Before HBO Treatment	After HBO Treatment
LL	0.5688	0.1722
HF	0.8684	0.6473
MG	0.5682	0.2907
TG	0.2741	0.4728
GE	0.6702	0.6330
$\bar{X}$±S.D.	0.5899±0.2148	0.4432±0.2093

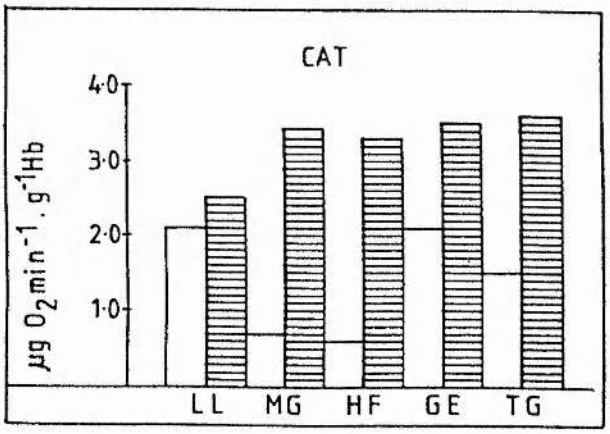
Table 34 Catalase of MS Patients Before and After HBO Treatment (expressed as $\mu gO_2\ min^{-1}\ g^{-1}$ Hb (n = 5)

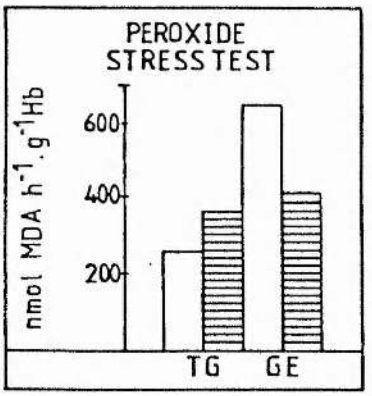
Patients	Before HBO Treatment	After HBO Treatment
LL	2.2323	2.4552
MG	0.6965	3.4416
HF	0.5912	3.2980
GE	2.0919	3.4879
TG	1.5449	3.5863
$\bar{X}$±S.D. (LL omitted)	1.2311±0.7152	3.4534±0.1199

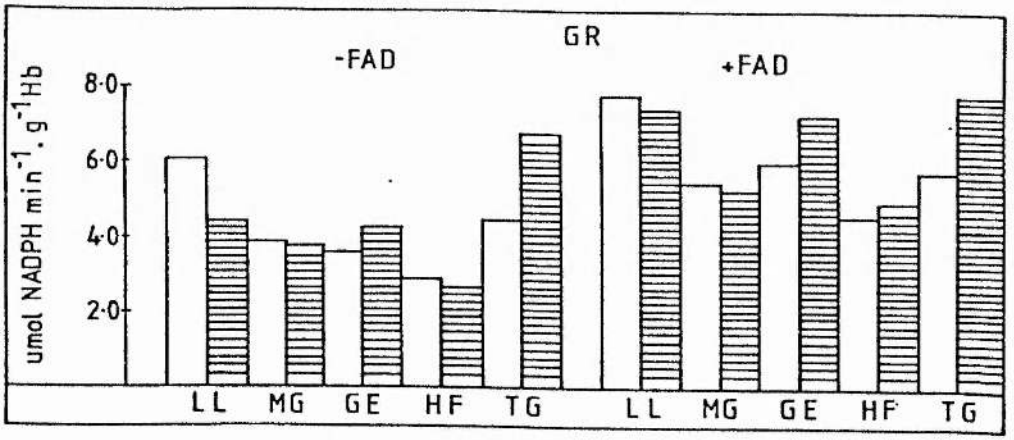
Fig. 34 Superoxide dismutase (SOD), MDA production, glutathione peroxidase (GPx), glutathione reductase (GR) and catalase (CAT) of erythrocytes from MS patients before  and after  hyperbaric oxygen treatment.


GPx
µmol NADPH min⁻¹.g⁻¹Hb
30
20
10
GE HF LL TG MG


SOD
mg SOD g⁻¹Hb⁻
0·8
0·6
0·4
0·2
LL HF MG TG GE


CAT
µg O₂ min⁻¹. g⁻¹Hb
4·0
3·0
2·0
1·0
LL MG HF GE TG


PEROXIDE
STRESS TEST
nmol MDA h⁻¹. g⁻¹Hb
600
400
200
TG GE


GR
-FAD
+FAD
umol NADPH min⁻¹. g⁻¹Hb
8·0
6·0
4·0
2·0
LL MG GE HF TG LL MG GE HF TG

From Table 30, 31, 32 and 33 and Fig. 34 it is clear that HBO treatment had no consistent effect on SOD, GR, GSH·Px or peroxidisability of red cells (Student's paired t-test, $P > 0.1$). Unless enzyme activities fluctuated very rapidly during and after treatment, it seems unlikely that HBO under the conditions defined here affects these particular enzymes in humans since four of the five patients, at the time of venepuncture had just (within 30 mins.) completed the 20th daily 90 mins. period in the chamber.

From Table 34 and Fig. 34 catalase activity is markedly increased ($P < 0.01$, Student's t test) by HBO in four out of five patients. It is significant that the one patient (LL) who showed only a moderate increase in catalase activity as a result of HBO had completed the 20 treatment course one week prior to blood sampling but had also received on 'top-up' treatment prior to venepuncture. Although based on one patient this may indicate that (i) induction and/or activation of catalase requires more than one 90 mins. treatment period and (ii) enzyme activity returns to near normal within one week of cessation of treatment. There is little literature on the effects of HBO on antioxidant enzymes in animals and humans but catalase was found to be HBO-inducible in fungi (Ahmad and Pritchard, 1970).

HBO has been shown to produce a 22% increase in lipid peroxidation (as measured by MDA release) in rat brain after exposure to 5 ATA pure O_2 for 5 mins., which returns to normal within 3 hrs of cessation of treatment

(Noda, McGeer and McGeer, 1983). If it is possible to compare these conditions of HBO to those used in this work and to extrapolate from rat to human brain then, considering the data in this work, it is possible to speculate that the long term protective effect of raised catalase may outweigh the potentially damaging increased lipid peroxidation which may accompany HBO. Interestingly, exogenous catalase, but not superoxide dismutase, is reported to offer protection against HBO-induced convulsion in mice (Hilton, Brown and Proctor, 1980), so that this enzyme may be generally of greater importance than other antioxidant systems in the CNS.

It would be of interest for future researchers in this area to undertake a more detailed study of the activity of these antioxidant enzymes, especially catalase, with time after HBO treatment to get a more accurate picture of the kinetics of any induction process.

SUMMARY

(1) Duchenne Muscular Dystrophy (DMD)

Muscle fibres and erythrocytes in DMD exhibit many abnormalities of membrane-associated properties which have led to the 'Membrane Hypothesis'. The Membrane Hypothesis states that the primary lesion in DMD involves a generalized defect in the plasma membrane. Since phospholipids play a crucial part in the structure, organization and function of membranes, subtle changes in these lipids could well be responsible for the observed abnormalities.

One possible alteration is a perturbation of the asymmetric distribution of phospholipids in the plasma membrane. Such asymmetry may be studied in erythrocytes using phospholipases. In this work, bee venom phospholipase A_2 was used to treat intact cells and derived ghosts. Two-dimensional TLC was used to separate the extracted lipids, which were quantified by phosphorus assay. Results showed the lipid composition is normal but the degradation of PC in DMD erythrocytes was higher and the differences were highly significant ($P < 0.01$). Increased PC degradation in erythrocytes may be explained in at least two ways: (i) transbilayer translocation of PC occurs more readily during the course of the experiment, or (ii) more PC is localized in the outer leaflet. If the results can be explained by (i) then spectrin, which is essential in maintaining lipid asymmetry, may be of abnormal structure and in fact, abnormalities of spectrin in DMD erythrocytes

have already been reported. If the explanation for the results is (ii), lipid rearrangement and changes in viscosity and fluidity can be expected, which would also result in abnormalities in spectroscopic data, osmotic fragility, ion transport and enzyme activities reported in DMD erythrocytes.

Na^+-K^+ ATPase activity of normal erythrocytes was reported to become 'Duchenne like' after incubation of cells with DMD plasma. A circulating factor from necrotic muscle was proposed to be responsible. The effect of DMD plasma on the asymmetry of membrane lipid was investigated but results showed that DMD plasma did not result in altered asymmetry in control cells. So the factor, if one exists, is not responsible for the increased PC degradation found in DMD erythrocytes and also the change in Na^+-K^+ ATPase activity is not due to a change in lipid asymmetry of the erythrocytes.

(2) Multiple Sclerosis (MS)

MS is a demyelinating disease of unknown cause. A dietary defect and abnormal immune response have been proposed. Lipid organization of the membrane of MS erythrocytes was investigated in this work to explore whether the reported abnormal physical properties in MS erythrocytes were due to abnormal lipid asymmetry. Results showed the membrane lipid composition and asymmetrical organization of MS is normal. So the reported increased cell size, increased osmotic fragility and reduced electrophoretic mobility of erythrocytes as well

as increased platelet stickiness in MS patients is not due to changes in lipid organization in the MS erythrocyte membrane.

Glutathione peroxidase, one of the enzymes which protects against membrane lipid peroxidation was reported to be decreased in MS erythrocytes. This might result in increased subsceptibility to lipid peroxidation of MS erythrocytes. The peroxidisability (using H_2O_2 as stressor) and the activities of the four enzymes, glutathione peroxidase, glutathione reductase, catalase and superoxide dismutase, were investigated. Results showed the peroxidisability of MS erythrocytes was significantly decreased ($P < 0.001$) which indicates that the membrane lipids of MS erythrocytes are less susceptible to peroxidation. This may be due to increased content of antioxidants (e.g. vitamin E, etc.) in the cells since the activities of the antioxidant enzymes were found to be normal in this work.

Hyperbaric oxygen (HBO) treatment has been proposed for treatment of MS patients. The effect of HBO treatment on the four antioxidant enzymes were investigated. Only catalase activity was found to be increased in erythrocytes of MS patients after HBO treatment. So raised catalase may be an important effect and outweigh the potentially damaging increased lipid peroxidation which may also accompany HBO treatment.

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APPENDIX

The following publications have been produced from the materials in this thesis:

1. 'Is erythrocyte membrane phospholipid organization abnormal in Duchenne muscular dystrophy?'
   M.I.S. Hunter, M.S. Lao and P. de Vane.
   Clinica Chimica Acta, 128: 69-74, 1983.
2. 'Erythrocyte membrane glycerophospholipid organization is normal in multiple sclerosis'.
   M.I.S. Hunter, M.S. Lao and D.L.W. Davidson,
   Neurochemical Research, has accepted for publication.
3. 'Erythrocyte antioxidant enzymes in multiple sclerosis and the effect of hyperboric oxygen'.
   M.I.S. Hunter, M.S. Lao, S.S. Burtles and D.L.W. Davidson,
   Has been submitted for publication.